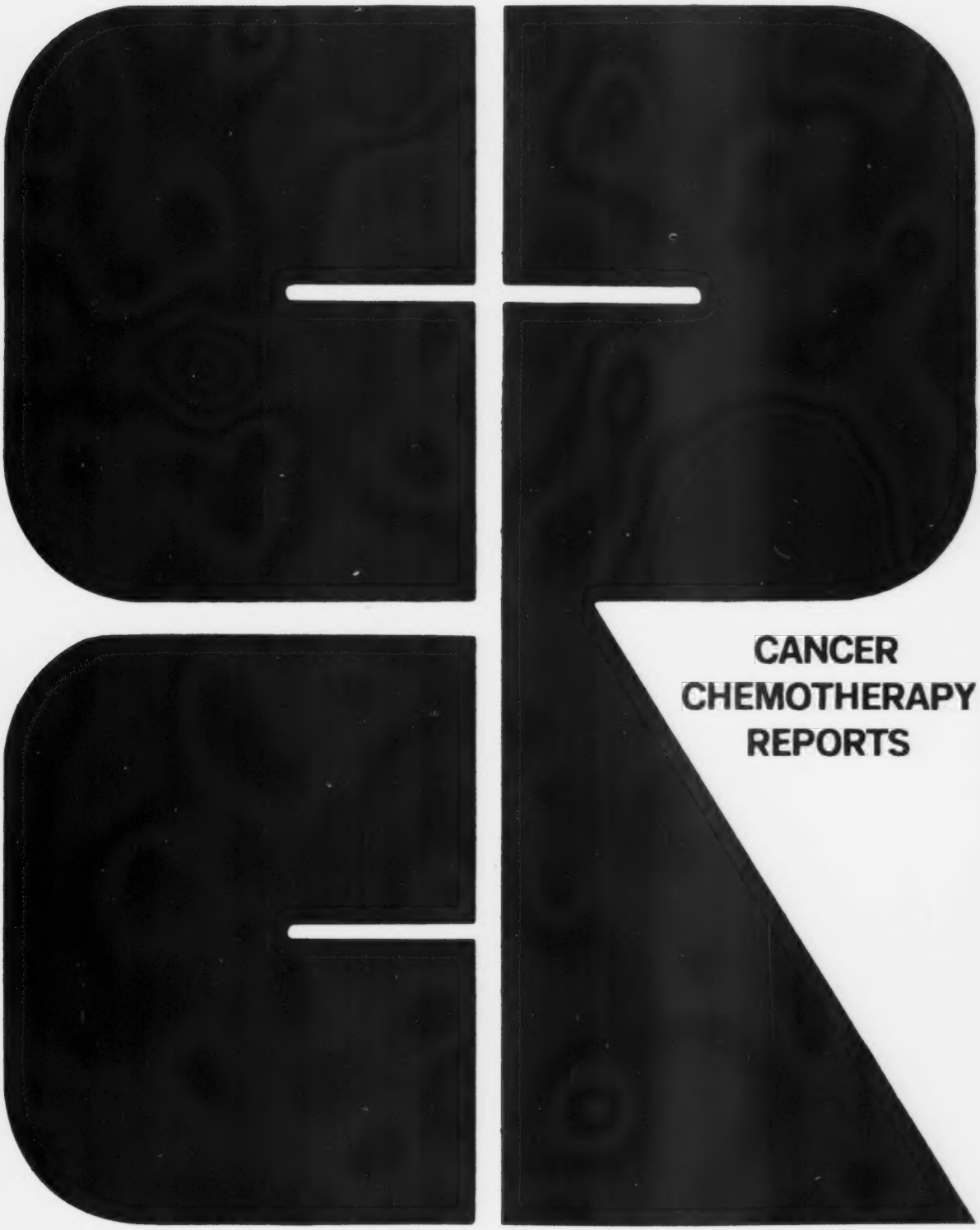

CANCER CHEMOTHERAPY REPORTS

Part 1

Mar/Apr 1974
Vol. 58, No. 2

Cancer Chemotherapy Reports

National Cancer Institute

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The Secretary of Health, Education, and Welfare has determined that the publication of this periodical is necessary in the transaction of the public business required by law of this Department. Use of funds for printing this periodical has been approved by the Director of the Office of Management and Budget through November 30, 1976.

LETTERS

Possible Inhibitory Effect of Androgens on Bone Marrow Suppression by Cytotoxic Agents—A Dose-Dependent Phenomenon? [1]

The recent report by Reyes et al (*Cancer Chemotherapy Reports* April 1973 [1]) on the use of fluoxymesterone to modify the bone marrow toxicity of BCNU was of considerable interest to us. We recently treated a patient with choriocarcinoma who had unusual resistance to bone marrow toxicity during the administration of high-dose combination chemotherapy. Of interest was the marked elevation of the patient's serum testosterone levels throughout the course of treatment.

Brief case report.—A 22-year-old Chinese man in pulmonary crisis with massive lung metastases secondary to choriocarcinoma was presented for treatment. The diagnosis was confirmed by lung biopsy and by the presence of markedly elevated serum human chorionic gonadotropin levels. Combination chemotherapy (actinomycin D, vincristine, vinblastine, methotrexate, and cyclophosphamide) was started and the patient had a partial response after 6–8 weeks of therapy (fig 1). Although severe stomatitis and marked alopecia developed after 4 weeks of treatment, there was no significant depression of bone marrow function. During this time, the patient was found to have persistently elevated serum testosterone and estrogen levels (fig 2). Terminally, brain metastases developed and treatment with large doses of dexamethasone was started. Coincidentally, the patient's serum testosterone levels fell and thrombocytopenia developed for the first time. (Recently, Ettinger et al demonstrated that large doses of dexamethasone may lower testosterone levels in hirsute women [2]. Whether or not a similar mechanism was operative in this patient is, of course, speculative.)

Since 1964, Brodsky et al have advocated the use of androgens to protect against bone marrow depression during cytotoxic chemotherapy (3). Although it is well known that androgens stimulate erythropoiesis, their "stimulatory" effect on leukopoiesis and thrombopoiesis is not as well appreciated. Recent reports by Wimer et al (4) and Horn et al (5) have suggested that androgens *in large doses* can increase leukopoiesis and thrombo-

[1] Chemical nomenclature, NSC numbers, and CAS registry numbers are included in the List of Compounds at the end of the text.

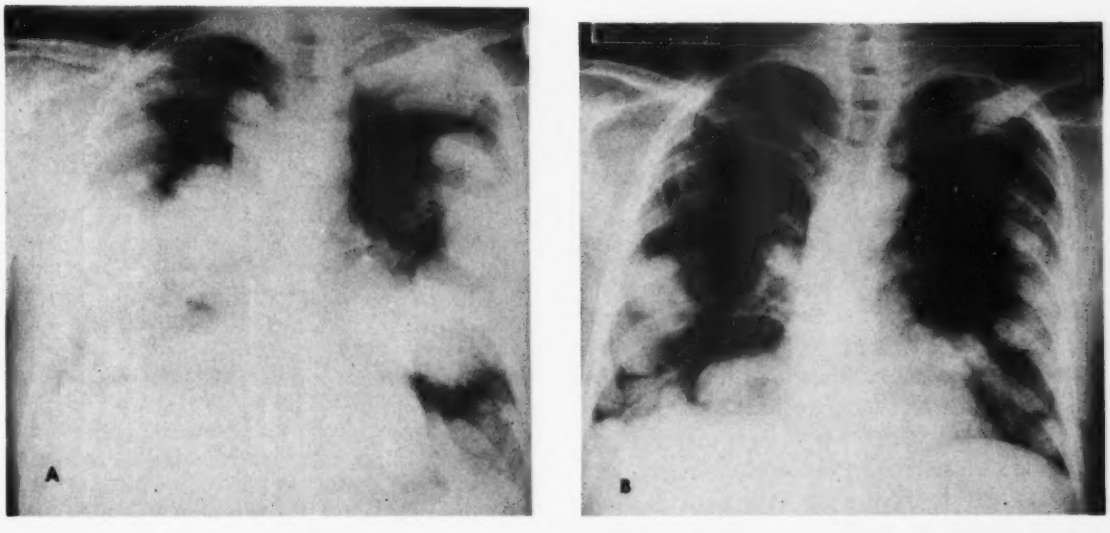

FIGURE 1.—*A*. Massive pulmonary metastases from choriocarcinoma. *B*. Partial regression after 2 months of combination chemotherapy with four drugs.

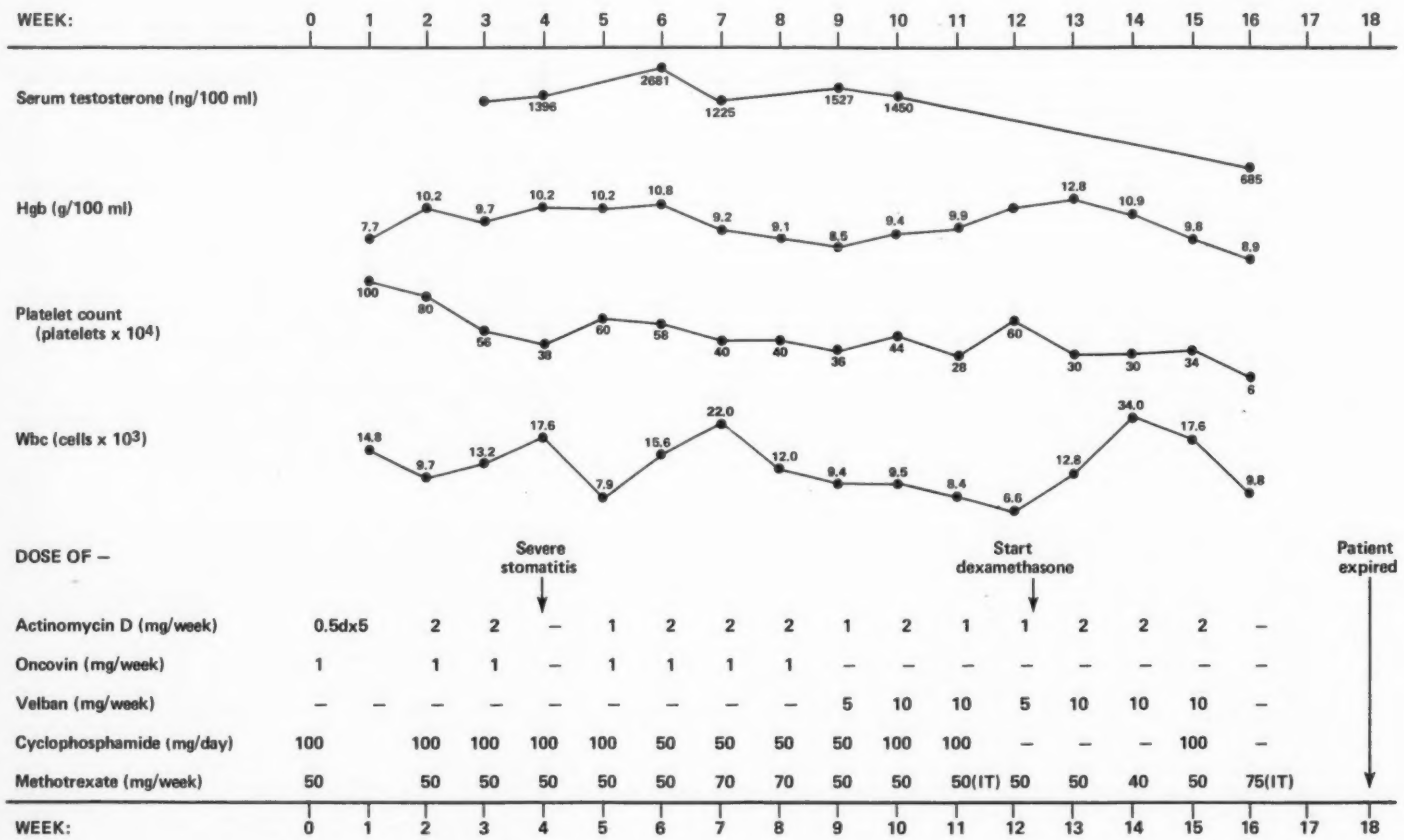

FIGURE 2.—Fluctuations of serum testosterone and hemoglobin (hgb) levels and platelet and white blood cell (wbc) counts during combination chemotherapy with actinomycin D, vincristine (Oncovin), vinblastine (Velban), cyclophosphamide, and methotrexate. Serum testosterone levels were determined by the method of Coyotupa et al [Analytic Letters 5:329, 1972] with modification using paper chromatography by Radio Assay Systems Laboratory (Carson, Calif). The normal value for serum testosterone in adult males is 300–1000 ng/100 ml. IT = intrathecally.

poiesis in humans. Reyes et al used fluoxymesterone at a dose of 30 mg/day (given orally) and observed no protective effect on bone marrow function, whereas Horn et al used calusterone at a dose of 200 mg/day (given orally) and Wimer et al used 17β-testosterone cypionate and testosterone enanthate at a dose of 600–800 mg/week (given intramuscularly [im]).

Our patient had unusual resistance to bone marrow toxicity during combination chemotherapy; this effect could *possibly* have been related to high endogenous testosterone levels in his circulating blood. We have since given oxymetholone at a dose of 60–100 mg daily to two patients with cancer and have been unable to demonstrate elevated blood testosterone levels or bone marrow protection from cytotoxic therapy. More recently we have used much larger doses of plain testosterone cypionate at a dose of 1200 mg/week (given im), and preliminary results suggest that bone marrow tolerance to cytotoxic therapy may be increased. Thus, if androgens *do* shield the bone marrow from the effects of cytotoxic drugs, it may be a dose-dependent phenomenon.

LIST OF COMPOUNDS

Actinomycin D: NSC-3053

Calusterone: NSC-88536; CAS reg. No. 17021-26-0; androst-4-en-3-one, 17β-hydroxy-7β,17-dimethyl-

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate

Dexamethasone: NSC-34521; CAS reg. No. 50-02-2; pregna-1,4-diene-3,20-dione, 9-fluoro-11β,17,21-trihydroxy-16α-methyl-

Fluoxymesterone: NSC-12165; CAS reg. No. 76-43-7; androst-4-en-3-one, 9-fluoro-11β,17β-dihydroxy-17-methyl-

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]-methylamino]benzoyl]-

Testosterone enanthate: NSC-17591; CAS reg. No. 315-37-7; androst-4-en-3-one, 17β-hydroxy-, heptanoate

Vinblastine: NSC-49842; CAS reg. No. 6449-03-2; vincaleukoblastine, sulfate (1:1), monohydrate

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

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4. Wimer BM, and Sloan MM. Remission of Felty's syndrome with long-term testosterone therapy. JAMA 223:671–673, 1973.
5. Horn Y, Halden A, and Gordan GS. The platelet-stimulating effects of 7-beta, 17-alpha-dimethyl-testosterone (calusterone). Blood 40:684–687, 1972.

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October 2, 1973

Should Ineffective Agents be Used in Combination Chemotherapy?

Kovach et al in their article entitled "Phase II Study of *Cis*-diamminedichloroplatinum (NSC-119875) in Advanced Carcinoma of the Large Bowel" (Cancer Chemother Rep 57: 357–359, 1973) show that "'*cis*-diamminedichloroplatinum appears to have little value in the treatment of colorectal adenocarcinoma." Yet, they suggest in the same paragraph that the drug *should* be used in trials of combination regimens. Do they wish to imply that drugs which are ineffective as single agents should be tried in combination for the same tumor? Or do they imply that the drug should

be used in combination therapy for tumors other than colorectal? If so, what is its effect used singly for the other tumors? I am unaware of any reports showing a beneficial effect of a drug used in combination when it is ineffective used alone. Consequently, I believe that when an agent is ineffective alone, its use in combination trials is generally unwarranted.

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December 3, 1973

COMMENTARY

Methodical Aspects of Cancer Chemotherapy After Radical Surgery [1]

A. M. Garin and M. R. Lichinicer [2]

Improvement of recurrence and survival data after surgery for cancer by means of adjuvant chemotherapy or immunotherapy is a worthwhile goal for the oncologist. A reasonable first step in the design of adjuvant therapy is to determine the clinical characteristics of the disease before and after surgery in both the individual patient and in the treatment group as a whole. It is advisable to single out patients with a good chance for cure as well as patients with a poor prognosis. Patients with the more typical development of this disease occupy a separate category.

To determine the "oncologic operability" of a patient we use established methods before the operation to discover metastases (scans of the liver and lymphatic nodes, angiography of the liver, cytologic study of the bone marrow, etc). After the operation other methods are used for assessing metastatic spread (search for tumor cells in the blood; study of hormones produced by the tumor [gonadotropin in metastases of choriocarcinoma of the uterus and testis; AKTG-like substance, antidiuretic, parathyroid, and other hormones in cancer of the lung, kidney, and ovary; catecholamines in neuroblastoma, etc]; determination of alpha fetoprotein in cancer of the liver and teratocarcinoma, and carcinoembryonic antigen in cancer of the colon; evaluation of the patient's delayed hypersensitivity status using dinitrochlorbenzol [DNCB] or tuberculin skin tests; and determination of the cytotoxic activity of lymphocytes).

Such detailed clinical disease characteristics form the basis for a reliable and rationale decision on an adjuvant therapy program which may be capable of improving significantly the results of an operation.

The second step in the study of surgical adjuvant therapy is to conduct comparative investigations after radical operation which allow the division of all patients into treatment groups for defining the effects of adjuvant therapy. Segregating patients as described above and then randomly assigning patients to various adjuvant therapies by the random-card method seems the best approach to test a given idea.

SELECTION OF METHODS

It is necessary to compare the treated group of patients with an untreated group of patients simultaneously admitted to the hospital during the search for successful adjuvant chemotherapy. Such control is necessary not only for the establishment of positive results but also to insure the earliest detection of detrimental treatment. For example, Brunner et al (1) showed an increase in the frequency of metastases in lung cancer as a result of the prolonged use of cyclophosphamide [3] after radical surgery. Similar results were observed in our investigation (2) after the administration of two courses of cyclophosphamide (3 g/dose) in patients with stage I and stage II breast cancer. These patients were under 50 years of age and had functional ovaries.

SELECTION OF DRUG

The selection of a drug for postoperative treatment is one of the most important aspects of the adjuvant therapy for cancer. Such se-

[1] Received Dec 18, 1972; revised Aug 30, 1973; accepted Oct 25, 1973.

[2] Institute of Experimental and Clinical Oncology, USSR Academy of Medical Sciences, Moscow.

[3] Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are included in the List of Compounds at the end of the text.

lection is performed using data from experimental studies and clinical trials. Major importance is attached to the effectiveness of a drug or a combination of drugs in patients with metastatic cancer. For instance, the following drugs have proved effective in the treatment of metastatic breast cancer: thioTEPA (40% response), cyclophosphamide (22%–60%), methotrexate (45%), vincristine (22%), ftorafur (45%), asaley (30%), as well as other drugs. In one study, the combination of vincristine, 5-fluorouracil, methotrexate, cyclophosphamide, and prednisolone caused complete or partial remissions in 85% of patients with metastatic breast cancer (3). The use of methotrexate, actinomycin D, 6-mercaptopurine, and daunorubicin led to tumor regression and cure in many patients with choriocarcinoma of the uterus. Similar data have been obtained in metastases of Wilms' tumor after treatment with actinomycin D, vincristine, and cyclophosphamide.

Significant tumor regression may occasionally be achieved in cancer of the lung (cyclophosphamide, methotrexate, and 1-methyl-1-nitrosourea), in cancer of the ovaries (thioTEPA, cyclophosphamide, chlorambucil, 5-fluorouracil, vinblastine, asaley, and sarcolysin), in cancer of the stomach and colon (5-fluorouracil, ftorafur, CCNU, and methotrexate), in cancer of the tongue and oral cavity (methotrexate, 5-fluorouracil, bleomycin, and hydroxyurea), in melanoma (5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide, BCNU, and 1-methyl-1-nitrosourea plus vincristine plus actinomycin D), in seminoma and teratocarcinoma of the testis (sarcolysin, cyclophosphamide, olivomycin A, mitomycin C, and vinblastine plus bleomycin), in sarcoma of the soft tissues and osteogenic sarcoma (methotrexate and adriamycin), in neuroblastoma in children (vinblastine, cyclophosphamide, and daunorubicin), and in hypernephroma (fluorobenzotepa).

Despite considerable success in the treatment of some patients with metastatic cancer, the overall response rate is 20%–30% for most drugs; rates of 50% or more occur infrequently. The rationale for selecting a drug for postoperative study is not always clear. It is clear, however, that there is little if any chance for effectiveness in most patients who receive postoperative chemotherapeutic treatment with a drug that has been shown to be marginally (20% or so) effective. In addition, the patients who do respond suffer from toxic manifestations (especially during prolonged treatment).

It would appear that drugs or combinations of drugs that are at least 50% effective in patients with metastatic cancer offer the best hope of successful adjuvant chemotherapy. At the same time, the prophylactic activity of a drug with less activity can also be studied. Two methods are used for this type of study: (*a*) the establishment of the drug's activity in tumor tissue cultures of the potential patient's own tumor, and (*b*) preoperative chemotherapy. If a drug shows in vitro antitumor activity the patient should be included in the trial. Then the division of the patients with sensitive tumors into two groups is made (those with postoperative treatment and those without postoperative treatment). Such an approach will help to establish the actual correlation of the drug's activity in vitro and in vivo.

Preoperative chemotherapy can be used with highly active drugs (more than 50% effective) and less active drugs (less than 50% effective). The attempt to exclude patients resistant to drugs from prophylactic chemotherapy is the main aim of this approach. Thus, if a patient shows no measurable response to a given drug prior to surgery, there seems little likelihood that that drug would have value as a surgical adjuvant in the same patient. At the same time, the success of a given regimen preoperatively does not guarantee its adjuvant value. That is why we evaluate these categories of patients separately.

Preoperative treatment can be carried out using marginally effective drugs (only a 20% activity rate) if the drug, when effective, produces significant tumor regression (ie, a decrease in size of greater than 50%). The following circumstances should be taken into consideration while conducting a "preoperative test."

(1) Only cases with a definite histologic

diagnosis should be included in the study.

(2) Only cases with measurable disease should be studied.

(3) Drugs without proven efficacy in metastatic disease should not be evaluated in adjuvant trials.

(4) Drugs or doses of drugs which complicate surgical treatment (eg, mitomycin C causes hypocoagulation, 5-fluorouracil causes a decrease of regeneration, etc) should not be used.

(5) It is desirable to estimate the immunodepressive activity (improbable in a short course of therapy), the interaction with anesthetic agents, and other pharmacologic effects of a drug proposed for adjuvant study in preliminary experimental and clinical investigations.

(6) It is expedient to treat the patients for no more than 2–3 weeks with 50%–75% of the median normal course dose.

SELECTION OF THERAPY STARTING DATE

The time of the beginning of postoperative therapy may be of great significance. The circulation of blood-borne tumor cells sharply increases during surgery and the conditions are established for their penetration into tissues (the establishment of cancer embolus in connection with hypercoagulation, decrease of tissue resistance, and other less studied factors).

The correlation of the number of circulating cells and the potential for the development of metastases have been proved by experimental and clinical studies. Repeated attempts to treat with cytostatic agents during the operation or very soon after the operation were made. There are contradictory conclusions on this question in Wilms' tumor and cancer of the breast, stomach, and lung, which show either minimal effect (on prognosis) or noneffectiveness of such early treatment. It should be admitted that the administration of thio-TEPA, 5-fluorouracil, cyclophosphamide, actinomycin D, or mechlorethamine during an operation does not have a desirable effect. Moreover, some of these agents (ie, mechlorethamine) have increased postoperative mortality in some studies.

In the future, however, attempts to influence the circulating malignant cells must be carried out. Preference must be given to drugs with quick, direct, highly effective, less toxic actions. The cytostatic agents must be applied in combination with drugs of different activity (eg, with rational doses of anticoagulants, etc).

A month after the operation is the usual time for us to begin postoperative adjuvant chemotherapy. By this time, postoperative complications disappear, the side effects of anesthesia are gone, and the patient's general state of health is optimal. The necessity for early (1 month after operation) drug therapy is motivated by the frequent existence of metastases, which tend to grow by this time. It is supposed that a comparatively low number of tumor cells can provide for a maximum therapeutic effect of the drug.

Conditions preventing the development and growth of metastases may appear in the organism after the removal of the tumor. The removal of the tumor in hypernephroma occasionally results in the disappearance of frank metastases. It was shown in a number of investigations, that the removal of the tumor leads to a disappearance of the factor which formerly blocked the cytotoxic action of the patient's lymphocytes on the tumor. The possibility of the immunologically detrimental action on tumor cells is apparent.

A study of delayed postoperative chemotherapy seems interesting in connection with the above discussion and with experimental data on the lack of effect of many drugs on "latent" tumor cells. In such cases, the time of the beginning of treatment for clinically similar patients is defined as three fourths of the time from the operation to the tentative average time to clinical detection of metastases. The group of patients with a possible early diagnosis of metastatic growth (the renewal of hormone secretion by tumor, the appearance of the blocked factor of serum, the appearance of the embryo-specific protein, etc) is of special importance.

The use of drugs in cases where these signs appear before the detection of metastases by usual methods may be called "early treatment." We are going to compare the effective-

ness of the so-called early prophylaxis, postponed prophylaxis, and early treatment.

DOSE AND REGIMEN

Doses and regimens of drugs are of prime importance. While administering a drug during operation it is desirable to choose a dose which does not cause undue toxicity.

Taking into account the established hematologic toxicity of thioTEPA, cyclophosphamide, 5-fluorouracil, and other drugs, the use of such drugs during the first month after operation should be carried out with a 50%–70% reduction in the usual dose. Subsequently, dosages may be escalated.

DURATION OF THERAPY

The duration of postoperative therapy may be of great importance for success. Insignificant achievements resulting from short prophylaxis regimens may be partly explained by the inaccurate assessment of the rapid destruction of the cancer cell population. Known achievements in the cure of lymphoblastic leukemia and Hodgkin's disease with long intensive chemotherapy show the inferiority of former chemotherapeutic concepts in oncology. Changing to an extended prophylactic use of actinomycin D (75 μg/kg every 3 months for 2 years) has increased the number of cures in children with Wilms' tumor.

The choice of duration of treatment for malignant tumors in adults has no scientific ground and is largely empirical. As a working hypothesis, one may use a time double that of the average expected time to the detection of metastases. Treatment may be either intermittent or continuous. Short intermittent courses are advantageous because they are convenient for the patients, they allow the use of outpatient treatment, they usually are less toxic, and they produce a relatively weak immunodepressive action.

CONCLUSIONS

Standardization of research concerning the treatment regimens and selection of patients, and the use of methods resulting from cooperative work will ensure the rapid solution of different practical and theoretical problems of cancer drug therapy. We are sure that the current prejudice against using antineoplastic drugs after surgical operation for prevention of metastatic growth is the result of ignoring methodical approaches to research. Collective experience, cooperative scientific programs, and optimism in research may unite surgeons and chemotherapists in studying the problem of postoperative drug therapy.

LIST OF COMPOUNDS

Actinomycin D: NSC-3053

Adriamycin: NSC-123127

Asaley: NSC-167780; CAS reg. No. 3577-89-7

BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-

Bleomycin: NSC-125066

CCNU: NSC-79037; CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-

Chlorambucil: NSC-3088; CAS reg. No. 305-03-3; butyric acid, 4-[*p*-[bis(2-chloroethyl)amino]phenyl]-

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate

Daunorubicin: NSC-83142; CAS reg. No. 23541-50-6

5-(3, 3-Dimethyl-1-triazeno)imidazole-4-carboxamide: NSC-45388; CAS reg. No. 4342-03-4

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU

Fluorobenzotepa

Ftorafur: NSC-148958; CAS reg. No. 17902-23-7; uracil, 5-fluoro-1-(tetrahydro-2-furyl)-

Hydroxyurea: NSC-32065; CAS reg. No. 127-07-1

Mechlorethamine: NSC-762; CAS reg. No. 55-86-7; diethylamine, 2,2'-dichloro-*N*-methyl-, hydrochloride; nitrogen mustard

6-Mercaptopurine: NSC-755; CAS reg. No. 6112-76-1; purine-6-thiol, monohydrate; 6-MP

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]-methylamino]benzoyl]-

1-Methyl-1-nitrosourea: NSC-23909; CAS reg. No. 684-93-5

Mitomycin C: NSC-26980; CAS reg. No. 50-07-7

Olivomycin A: NSC-76411

Prednisolone: NSC-9900; pregna-1,4-diene-3,20-dione, 11β,17,21-trihydroxy-

Sarcolysin: NSC-14210; alanine, 3-[*p*-[bis(2-chloroethyl)amino]phenyl]-, monohydrochloride, DL-; phenylalanine mustard

ThioTEPA: NSC-6396; CAS reg. No. 52-24-4; phosphine sulfide, tris(1-aziridinyl)-

Vinblastine: NSC-49842; CAS reg. No. 6449-03-2; vincaleukoblastine, sulfate (1:1), monohydrate

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

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Experimental and Clinical Studies of the Antitumor Activity of 1-Methyl-1-nitrosourea (NSC-23909) [1,2]

N. M. Emanuel, E. M. Vermel, L. A. Ostrovskaya, and N. P. Korman [3]

SUMMARY

Experimental and clinical studies of the antitumor properties of 1-methyl-1-nitrosourea have been carried out. The agent was effective against nine animal tumor systems. The effect of dose levels, route, and schedule of treatment on the drug's activity were also studied. The kinetics of the antitumor action of 1-methyl-1-nitrosourea against Ehrlich carcinoma was studied. The function

$$\sqrt[3]{N} = 1 + bt$$

fits the tumor growth in both control and treated animals. It was shown that daily treatment (20 mg/kg/day × 6 days) increased the doubling time of the tumor cell population by a factor of 2 and single treatment (120 mg/kg) increased it by a factor of 4.5 compared to control animals. A total of 140 adults with different advanced malignant neoplasms resistant to any other kind of therapy have received 1-methyl-1-nitrosourea. A considerable number of objective positive responses occurred among patients with undifferentiated carcinoma of the lung (18 of 21 patients treated) and Hodgkin's disease (15 of 18 patients treated). These responses were always accompanied by the subjective and symptomatic improvement of the patients. Drug infusions were followed by more or less immediate toxic reactions (vomiting, nausea) and myelotoxicity. The results obtained have encouraged us to recommend 1-methyl-1-nitrosourea for the palliative treatment of patients with undifferentiated carcinoma of the lung and Hodgkin's disease.

[Cancer Chemother Rep 58:135–148, 1974]

N-nitrosourea alkyl derivatives are a fairly new group of potential antineoplastic agents. Compounds such as 1-methyl-1-nitrosourea, BCNU, and CCNU have shown definite inhibitory activity against a wide spectrum of experimental tumor systems (1–5). BCNU and CCNU have been extensively tested for antineoplastic activity in many human neoplasms. Clinical benefit has been described in Hodgkin's disease, malignant melanoma, gastrointestinal neoplasms, various carcinomas, primary and metastatic brain tumors, the acute stage of chronic myelocytic leukemia, acute leukemia, and several other miscellaneous neoplasms (6–9).

[1] Received Dec 18, 1972; revised Aug 30, 1973; accepted Oct 25, 1973.

[2] Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are included in the List of Compounds at the end of the text.

[3] Institute of Chemical Physics, Academy of Sciences of the USSR, Moscow.

The antitumor properties and mechanisms of action of some alkyl-nitrosoureas, including methyl-, ethyl-, propyl-, and isobutyl-nitrosourea, have been investigated in our laboratory since 1964 (10–14). One of these compounds, 1-methyl-1-nitrosourea, was chosen in 1966 for clinical trial (13, 15, 16). The results of experimental and clinical studies of 1-methyl-1-nitrosourea are presented in this paper.

MATERIALS AND METHODS

The chemical structure of 1-methyl-1-nitrosourea is shown in figure 1; its molecular weight is 103. The drug was stored in 100–400-mg ampules of lyophilized compound at 5 C. The drug solutions in concentrations of 0.2%–2% in 0.9% NaCl were made at room temperature shortly before use.

Experimental Studies

Animals.—Randombred and inbred mice (20–22 g) and randombred albino rats (100–120 g) were used.

Toxicity.—The toxicity to mice was studied by administering the drug in single doses and daily doses for 5 days. The drug was given at various dose levels intravenously (iv), orally, intraperitoneally (ip), and subcutaneously (sc). Doses lethal to 50% of the animals (LD50) and maximum tolerated doses (MTD) were determined making use of standard methods (17). The tolerance of rats to 1-methyl-1-nitrosourea was also studied. The drug was given daily for 6 days in two doses (10 and 25 mg/kg orally). The body weight of every rat and the white blood cell counts (wbc) in the peripheral blood were measured before, just after, and 20 days after the treatment.

Animal tumors.—Tumor systems used in this study were Ehrlich carcinoma, Sarcoma 37, Sarcoma 180, and NK/Ly lymphoma (all of which were maintained in ascites form in randombred mice). Solid tumor strains included spontaneous mammary adenocarcinoma in A mice, Harding-Passey melanoma in C3H mice, Walker rat carcinosarcoma 256, and Sarcoma 45 in rats. Seven-day-old ascites tumors were transplanted ip with 6×10^6 tumor cells in 0.2 ml of inoculum. Solid tumors were maintained by sc implantation of tumor fragments into the subaxillary space.

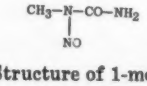

FIGURE 1.—Structure of 1-methyl-1-nitrosourea.

Antitumor evaluation of 1-methyl-1-nitrosourea.—The antitumor activity of 1-methyl-1-nitrosourea was assessed by the following: kinetics of tumor growth inhibition, the increase in median lifespan over controls, and the tumor weight inhibition (TWI) index expressed as percent of inhibition relative to untreated controls. The TWI was calculated as follows:

$$TWI = \frac{F_c - F_t}{F_c} \times 100$$

where F_c and F_t = the mean tumor weight or ascites volume in control and treated groups respectively.

Dosage and schedule.—The effects of dose levels and routes and schedules of treatment were studied in mice with Ehrlich carcinoma. 1-Methyl-1-nitrosourea was given in a single 50- or 100-mg/kg dose or daily for 5 consecutive days in 5-, 10-, 20-, or 50-mg/kg doses. Animals received the drug ip, sc, iv, or orally either immediately or 4 days after tumor implantation. 1-Methyl-1-nitrosourea was evaluated against other tumors and was given ip and sc daily for 5 days. Mice with ascites tumors and rats with Walker carcinosarcoma 256 were treated starting from Day 1 after tumor inoculation. Animals with other tumor strains were treated as soon as the palpable tumors appeared. Animals were decapitated 1 day after the last drug injection. The tumor weight or ascites volume was measured and the TWI index was calculated. Each dose level was given to a group of six to ten animals. The dose-response relationship was replicated independently at least three times at different periods. The "Student's t" test was used for statistical analysis ($P \leq 0.05$).

Kinetic Method of Data Analysis

Application of the kinetic method to the oncologic study allows a description of parameters which measure the growth of ascitic tumors, solid tumors, and leukemias, as well

as the regression and regrowth of tumors after chemotherapy. Many experimental tumor systems presently in use for chemotherapeutic evaluation have been studied for various growth parameters and described by mathematical models (18–21).

In our experiments, alkyl-nitrosoureas have been investigated (11–13,18), and kinetic data on the antitumor activity of 1-methyl-1-nitrosourea against Ehrlich carcinoma are considered below. The drug was injected sc both as a single 120-mg/kg dose and daily for 6 consecutive days at a 20-mg/kg dose. Treatment began 24 hours after tumor implantation.

To obtain data on tumor growth in both control and treated animals, three to five mice from the control and treated groups were decapitated daily starting 1 day after tumor inoculation. The volume of ascitic fluid and the total number of tumor cells in the peritoneal cavity of mice were measured according to the described procedures (12,19). Corresponding kinetic growth curves were plotted. Each point on the curve corresponds to nine to 15 animals.

The linear regression method was used for analysis of the experimental results and evaluation of the growth constants. A "Fisher F" statistical test indicated that the power function described later in this paper was a good representation of the experimental data (22).

The doubling time (DT) or the time required for the number of tumor cells to increase by a factor of 2 was calculated on the basis of the known kinetic model of tumor growth.

Clinical Studies

Patients.—A total of 140 adults with different advanced malignant neoplasms have received 1-methyl-1-nitrosourea to date. Diagnoses were proved histologically for 120 patients and cytologically for 20 patients. A total of 170 courses of treatment with the drug were carried out. Twenty-four patients received two courses and three patients received three courses of 1-methyl-1-nitrosourea.

Dosage and schedule.—After studying various methods of drug administration, the rapid iv injections were found to be most suitable. Infusions by drops during 20–30-minute periods were given to four patients and strongly increased the direct toxic effects (*see* below). Using oral administration, severe epigastric pains occurred in all four patients who received the drug in this way. These pains appeared after every drug administration and lasted several days. Complications accompanying intracavitary injections were the same as those noted with iv infusions. Sc and intramuscular (im) injections were not tried since experimental data have revealed that necrotic lesions appeared at the injection site (23). 1-Methyl-1-nitrosourea was tested in a wide range of single daily doses (from 20 mg to 2 g [0.5–35 mg/kg]), of total doses (from 160 mg to 12 g [4–220 mg/kg]), and of intervals between administrations (from 1 to 5 days). The optimal schedule of therapy seemed to be a single dose of 8–10 mg/kg given iv every 3 days. Total doses were selected on an individual basis depending on tolerance and treatment efficacy. The course of therapy usually consisted of eight to ten injections.

Antitumor evaluation of 1-methyl-1-nitrosourea.—The evaluation of antitumor effect was based on the results of the first course of 1-methyl-1-nitrosourea therapy. A decrease in the tumor size and metastasis determined by direct measurements or by roentgenographic findings was considered an objective response. The decrease in tumor size was estimated according to the grading system proposed by the Institute of Experimental and Clinical Oncology of the USSR Academy of Medical Sciences: 3 = more than 50% tumor regression for longer than 1 month; 2 = 25%–50% tumor regression for not less than 1 month; 1 = any tumor regression up to 25% for less than 1 month; 0 = no objective response. A decrease in the disease symptoms and an improvement in the patient's condition were considered a symptomatic and subjective response.

RESULTS

Animal Toxicity

The toxic doses and MTDs of 1-methyl-1-nitrosourea are listed in table 1. The toxicity was dependent upon the route of administration: iv and ip injections were found to be more toxic than oral and sc injections.

TABLE 1.—Effect of treatment route on 1-methyl-1-nitrosourea toxicity in mice

Route	Treatment schedule			
	Single dose		Five doses	
	LD50	MTD	LD50	MTD
	(mg/kg/day)			
iv	180	100	30	20
oral	200	100	80	50
ip	150	100	30	20
sc	200	150	80	50

Oral administration of 1-methyl-1-nitrosourea at a dose of 25 mg/kg daily for 6 days resulted in a weight loss of 14% and leukopenia. The wbc counts had not recovered to their normal levels 20 days after treatment. When the drug was administered at a dose of 10 mg/kg the rats gained weight and did not differ from normal animals. Leukopenia was not observed (table 2).

Animal Tumor Data

The kinetics of Ehrlich carcinoma growth in control and treated animals are shown in figure 2. Growth curves for the number of tumor cells (curve B, during an 11-day period) can be described by the following equation:

$$\sqrt[3]{N} = l + bt$$

where N = the number of tumor cells; $l = a - b\bar{t}$; a and b = linear regression coefficients; and $\bar{t}$ = time, when $\sqrt[3]{N} = a$.

The curve's linearity with respect to the coordinates $\sqrt[3]{N}$ and t confirmed that equation (1) fit the observed data.

Numerical values for growth constants and other quantitative characteristics of 1-methyl-1-nitrosourea antitumor action against Ehrlich carcinoma are given in table 3.

It can be seen in figure 2 and table 3 that the drug is somewhat effective when given on a daily treatment schedule but is more effective when given as a single treatment. The maximum number of tumor cells decreased considerably after the drug treatment: 150 × 10^6 cells were noted after single treatment and 350 × 10^6 cells were noted after daily treatment compared to 1000 ∧ 10^6 cells in control animals. It should be mentioned that after daily treatment with 1-methyl-1-nitrosourea (curve B), the number of tumor cells achieved its maximum value of 350 × 10^6 cells on the 12th day and then decreased to 150 × 10^6. The median lifespan of the mice increased by 35% and 20% after single and daily treatment respectively as compared to controls.

TABLE 2.—Effects of 1-methyl-1-nitrosourea given orally on body weight and wbc counts of intact rats

Dose (mg/kg/day)	No. of rats treated	Body weight (g)		Wbc counts (× 10^3 cells/mm^3)		
		Before treatment	After treatment	Before treatment	After treatment	20 Days after treatment
10 × 6	12	103	110	10.7	8.5	9.2
25 × 6	12	115	99	13.8	5.2	5.0
Untreated controls	16	108	115	14.0	15.2	15.6

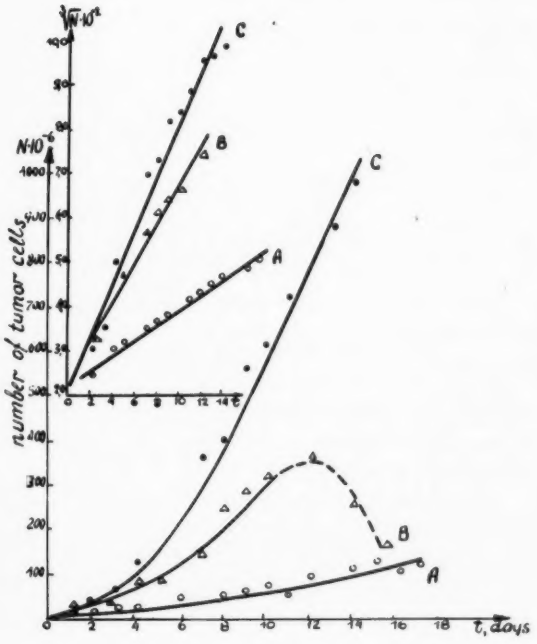

FIGURE 2.—Kinetic growth curve of total number of Ehrlich carcinoma tumor cells and their linear anamorphoses. *A*. 1-Methyl-1-nitrosourea single treatment at a dose of 120 mg/kg. *B*. 1-Methyl-1-nitrosourea daily treatment at a dose of 20 mg/kg/day. *C*. Control.

A knowledge of the function approximating the observed processes enables us to calculate the doubling time of the tumor cells in the following way: the differential equation of the progressive increase in the number of tumor cells can be shown as

$$\frac{dN}{dt} = \frac{\ln 2}{T} N$$

or in the logarithmic form as

$$\frac{d\ln N}{dt} = \frac{\ln 2}{T} . \quad (2)$$

Consequent logarithmation and differentiation of equation (1) result in the following relationship:

$$\frac{d\ln N}{dt} = \frac{3b}{1 + bt} . \quad (3)$$

If equations (2) and (3) are assumed to be equal, the doubling times can be determined as follows:

$$DT = 0.23\left(t + \frac{1}{b}\right). \quad (4)$$

It is evident that doubling time is a function of time or tumor size and increases at a constant rate as follows:

$$\frac{dDT}{dt} = 0.23 .$$

Changes in the doubling time of Ehrlich carcinoma cells in control and treated animals are shown in figure 3. Corresponding equations are given in table 3. These data show that daily treatment increases the doubling time by a factor of 2 and single treatment increases it by a factor of 4.5.

A comparative study of the effects of route and schedule of administration on 1-methyl-1-nitrosourea activity against Ehrlich carcinoma (%TWI) is shown in table 4. The activity varied somewhat for different routes of treat-

TABLE 3.—Quantitative characteristics of 1-methyl-1-nitrosourea antitumor activity against Ehrlich carcinoma

Dose (mg/kg/day)	Treatment schedule	Median lifespan (days ± SD)	Latent period for ascitic fluid (days)	Maximum ascites volume (ml)	Maximum No. of tumor cells	Equations for the tumor cell growth curves	Tumor cell doubling times
120	Day 1 only	19.0 ± 5.4	7	7.8	150×10^6	$\sqrt[3]{N} = 2.26 + 0.17t$	$0.23\ (t + 13.4)$
20	Daily, Days 1–6	16.9 ± 1.9	6	4.8	350×10^6	$\sqrt[3]{N} = 2.46 + 0.43t$	$0.23\ (t + 5.7)$
Untreated controls	—	13.9 ± 1.7	3	10.2	1000×10^6	$\sqrt[3]{N} = 1.89 + 0.63t$	$0.23\ (t + 2.98)$

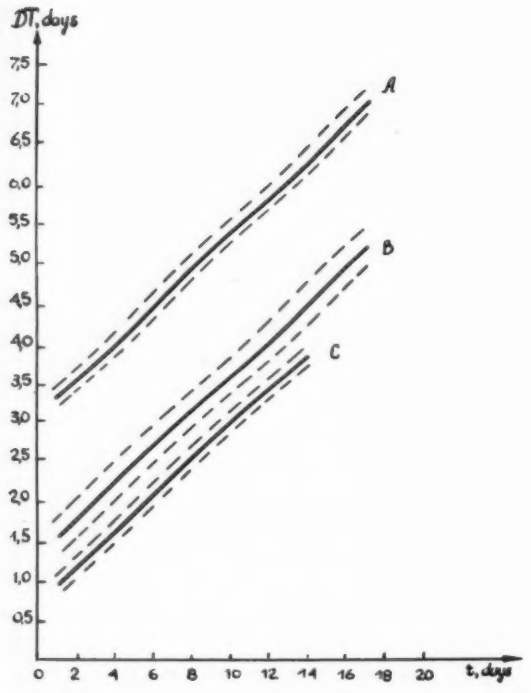

FIGURE 3.—Effects of 1-methyl-1-nitrosourea on changes in doubling time for Ehrlich carcinoma tumor cells. *A*. 1-Methyl-1-nitrosourea single treatment at a dose of 120 mg/kg. *B*. 1-Methyl-1-nitrosourea daily treatment at a dose of 20 mg/kg/day. *C*. Control.

ment (ip, iv, oral, sc). When therapy began the day after tumor inoculation, 1-methyl-1-nitrosourea inhibited tumor growth by 80%–90% both on daily treatment (for 5 days) and on single treatment. The efficacy of 1-methyl-1-nitrosourea was less if the therapy was started on the fifth day after transplantation. As seen in table 4, the drug seemed to be more effective on single treatment than on daily treatment.

Data on the antitumor activity of 1-methyl-1-nitrosourea against some other animal tumors (calculated as %TWI) are given in table 5.

The tumors most responsive to 1-methyl-1-nitrosourea were Sarcoma 45 and Walker carcinosarcoma 256. Their growth was inhibited by 60%–85% even with daily doses as small as 5 mg/kg ip or 10 mg/kg sc. Higher doses inhibited the growth of these tumors by 90%–95%.

The growth of spontaneous mammary carcinoma, NK/Ly lymphoma, Sarcoma 180, and Sarcoma 37 was also inhibited by 1-methyl-1-nitrosourea. Daily doses of 20 or 10 mg/kg ip and 50 or 20 mg/kg sc inhibited tumor growth by 70%–90%.

Harding-Passey melanoma and Adenocarcinoma 755 were inhibited much less by this drug. Their growth was inhibited by no more than 65% when the MTD of the agent was given.

Thus, these investigations with 1-methyl-1-nitrosourea have shown that this agent is effective against several animal tumors and that in therapeutic doses it is not profoundly toxic. These data allowed us to proceed with a clinical evaluation of 1-methyl-1-nitrosourea.

TABLE 4.—Effect of treatment route and schedule on antitumor activity of 1-methyl-1-nitrosourea in Ehrlich carcinoma

Treatment schedule	Dose (mg/kg/day)	Treatment route			
		ip	sc	oral	iv
		(percent of inhibition)			
Day 1 only	100	90	90	90	95
	50	85	75	60	50
Daily, Days 1–5	20	90	90	70	65
	10	75	80	50	70
Day 5 only	100	90	85	70	90
	50	45	50	65	40
Daily, Days 5–10	20	65	70	60	40
	10	—	60	—	0

TABLE 5.—Antitumor activity of 1-methyl-1-nitrosourea against some experimental tumor systems

Dose (mg/kg/day) and treatment schedule	Treatment route	Tumor growth inhibition (%)							
		Walker carcinosarcoma 256	Sarcoma 45	Spontaneous mammary adenocarcinoma	Sarcoma 180	Sarcoma 37	NK/Ly lymphoma	Harding-Passey melanoma	Adeno-carcinoma 755
20, Daily × 5	ip	95	95	90	90	90	85	65	60
10, Daily × 5	ip	90	90	65	80	70	40	50	40
5, Daily × 5	ip	80	80	—	60	60	—	—	—
50, Daily × 5	sc	—	—	—	85	70	90	—	—
20, Daily × 5	sc	95	90	70	75	60	70	55	60
10, Daily × 5	sc	60	85	—	30	0	40	—	—

Clinical Trials: Toxicity

The side effects observed after the clinical application of 1-methyl-1-nitrosourea and their frequency are listed in table 6. Some of the toxic reactions appeared immediately after the drug injection and others developed after a specific total dose of the drug had been given.

Nausea and vomiting were observed in almost all patients, especially after the first injection. The patients usually felt sudden nausea 20–40 minutes after the injection followed almost immediately by vomiting which lasted for several minutes. Repeated vomiting (2–3 hours) was noted in some patients. Subsequent drug injections were followed by nausea and vomiting for most patients, but one third of these patients did not have vomiting, and nausea was less than after the first injection. Infusions by drops were also followed by nausea and vomiting.

Nausea and vomiting appeared after any single drug dose and were more severe and prolonged after higher doses. When the drug was injected with 2–3-day intervals, nausea disappeared in most cases, appetite improved, and the patients tolerated the treatment course better than when the drug was injected daily.

By the end of the course (seven to eight injections) most patients had anorexia, asthenia, and disagreeable mood which sometimes caused interruption of the treatment although there were no other serious side effects. These side effects quickly disappeared after the treatment ended.

Diarrhea appeared suddenly 20–40 minutes after injection in 29% of the patients. Diarrhea usually occurred once and was severe.

TABLE 6.—Side effects of 1-methyl-1-nitrosourea treatment

Toxic reaction	Percent of occurrence
Nausea	95.6
Vomiting	85.8
Diarrhea	29.0
Leukopenia	21.8
Thrombocytopenia	19.2
Allergy	10.6
Occluded veins, phlebitis	9.5

The contact of 1-methyl-1-nitrosourea drops on the skin led to painless pigmentation which disappeared in 2–5 weeks. Pigmentation was avoided by immediately washing the affected area with water.

Iv injections of 1-methyl-1-nitrosourea should be performed with care; when the drug got under the skin it led to hyperemia, infiltration, and sometimes necrotic lesions of the skin and subcutaneous tissues. These phenomena completely reversed after 1–4 weeks of the conventional treatment.

The drug had myelotoxic activity—22.5% of the patients had leukopenia and thrombocytopenia. The nadir of leukocytes was between 2000 and 3000 cells/mm^3 for 12 of 33 patients (36.4%), between 1000 and 2000 cells for ten patients (30.3%), and less than 1000 cells for 11 patients (33.3%). The nadir of thrombocytes was greater than 75,000 platelets/mm^3 for seven of 29 patients (24.1%), greater than 50,000 platelets for seven patients (24.1%), and less than 50,000 platelets for 15 patients (51.8%). It should be noted that almost all cases of severe hematopoietic depression occurred in patients previously treated with both radiation therapy and chemotherapy. Both leukopenia and thrombocytopenia were observed in most patients with hematopoietic depression, although six had only leukopenia and one had only thrombocytopenia.

Hematopoietic depression after treatment with 1-methyl-1-nitrosourea began at different times after the beginning of treatment, usually after 3–4 weeks, ie, after different total doses of the drug (20–120 mg/kg). When hematopoietic depression was observed, treatment was interrupted. Leukopenia and thrombocytopenia lasted for 3–30 days after treatment was stopped and in most patients they lasted no longer than 2 weeks. Thrombocytopenia usually developed and disappeared several days later than leukopenia, and it was never followed by hemorrhage. No delayed hematopoietic depression was noted after the drug treatment.

Six of the above 33 patients displayed the hematopoietic depression as severe pancytopenia which was irreversible and caused the death of these patients. Analysis showed that these patients received very high doses of the

drug (15–28 mg/kg) and, in addition, they had previously received intensive radiation therapy and chemotherapy.

The myelotoxic action of 1-methyl-1-nitrosourea depends on the level of the single drug dose. As seen in table 7, the average single dose was greater in patients with leukopenia and thrombocytopenia than in those without hematopoietic depression, although the total doses for these groups of patients were the same.

The dependence of 1-methyl-1-nitrosourea myelotoxicity on the level of the single drug dose is also proved by the fact that hematopoietic depression was noted in 13 (14.9%) of 87 patients who received no more than 10 mg/kg (6–10 mg/kg) as a single dose and in 21 (32.8%) of 64 patients who received the drug in doses greater than 10 mg/kg (11–28 mg/kg).[4]

An analysis of the relationship between 1-methyl-1-nitrosourea doses and the nadir of hematopoietic depression showed that there was no distinct correlation between the doses and the degree of myelotoxic action. Severe hematopoietic depression was observed at different doses and in several instances even at low doses. At the same time, it should be noted that leukopenia and thrombocytopenia often did not appear when rather high doses were given. The myelotoxic effect of 1-methyl-1-nitrosourea is undoubtedly controlled by the individual peculiarities of the patient and his physical condition during the treatment.

Sixteen patients exhibited side effects similar to allergic reactions, consisting of cutaneous pruritus and urticaria. These phenomena were not too serious and reversed soon after the end of the treatment and the application of antihistamines.

[4] In 19 courses 1-methyl-1-nitrosourea was applied in doses less than 6 mg/kg.

In 14 patients the iv injection of 1-methyl-1-nitrosourea led to induration of the vein in which the drug was repeatedly injected, often with skin pigmentation along this vein. Phlebitis developed in four patients; occluded veins occurred in 11 patients.

Clinical Trials: Antitumor Activity

Tumor type, localization of the disease, and response to 1-methyl-1-nitrosourea are shown in table 8. The treatment efficacy could be evaluated for 122 patients. Data were excluded on other patients who received an insufficient dosage of the drug during the early stages of the investigation.

The data given in table 8 show that the various degrees of objective response were observed in 38.5% of the patients and symptomatic and subjective responses in 14.7%; in all cases objective responses were accompanied by patient improvement.

The best results were noted in patients with carcinoma of the lung and Hodgkin's disease.

Comparative data on 1-methyl-1-nitrosourea treatment of patients with carcinoma of the lung and the histologic type of the tumor are given in table 9. The best responses to the drug were observed in patients with undifferentiated carcinoma of the lung (small cell). Objective positive responses were recorded in 18 of 21 treated patients (85.7%); the tumor regression was more than 50% for eight patients and 25%–50% for six patients. Two of these 21 patients had been previously treated with cyclophosphamide (4–5 months before the 1-methyl-1-nitrosourea therapy) without effect. The 1-methyl-1-nitrosourea treatment resulted in objective improvement. Two of the three patients who were treated with 1-methyl-1-nitrosourea without effect had previously received lobectomy but lung tumor regrowth

TABLE 7.—Average single and total doses of 1-methyl-1-nitrosourea for patients with and without hematopoietic depression

Patient group	No. of courses	Average single dose (mg/kg)	Average total dose (mg/kg)
Without hematopoietic depression	117	10.2	79.1
With hematopoietic depression	34	13.4	74.2

TABLE 8.—Distribution of patients according to tumor site and response to 1-methyl-1-nitrosourea

Tumor site	No. of patients treated*	No. of objective responses	No. of subjective and symptomatic responses
Carcinoma, lung	57 (48)	23	9
Hodgkin's disease	19 (18)	15	1
Cancer, stomach	11 (9)	2	1
Carcinoma, cervix	8 (8)	1	0
Reticulosarcoma	8 (8)	2	1
Cancer, ovary	5 (2)	1	0
Carcinoma, rectum	4 (3)	3	0
Cancer, corpus uteri	3 (3)	—	—
Hypernephroma	2 (2)	—	—
Acute leukemia	3 (2)	—	—
Cancer, breast	2 (2)	—	—
Malignant melanoma	2 (2)	—	—
Malignant chordoma	2 (2)	—	—
Mesothelioma	2 (2)	—	—
Cancer, vulva	2 (2)	—	—
Cancer, nasopharynx	1 (1)	—	—
Multiple myeloma	1 (1)	—	—
Leukomyosarcoma, stomach	1 (1)	—	—
Chronic lymphocytic leukemia	1 (1)	—	—
Cancer, bladder	1 (1)	—	—
Cancer, colon	1 (1)	—	—
Chondrosarcoma	1 (1)	—	—
Soft tissue sarcoma	1 (1)	—	—
Hemangiopericytoma	1 (1)	—	—
Teratoblastoma, mediastinum	1 (1)	—	—
Totals	140 (122)	47	14

*Numbers in parentheses represent evaluable patients.

and metastases of the tumor in the lymphatic nodes had occurred.

Nineteen patients treated with 1-methyl-1-nitrosourea had considerable symptomatic and subjective improvement; ten of them could return to work for some time. The objective remissions lasted for 1–10 months and the median duration of remission was 2.4 months. The subjective improvement lasted for the same period of time. It should be emphasized that all of these patients had advanced neoplastic disease with metastases in the peripheral lymphatic nodes and in the lymphatic nodes of the lung roots and the mediastinum. Most patients had complete or partial atelectasis of the lung; some patients had a remarkable compression syndrome, specific pleurisy, or metastasis to other organs.

The responses of patients who had other histologic lung tumor types were considerably worse; positive effects appeared infrequently and were, as a rule, weaker.

TABLE 9.—Efficacy of 1-methyl-1-nitrosourea in various histologic types of carcinoma of the lung

Histologic type	No. of patients treated	No. of objective responses (grade) 3	2	1	0	No. of subjective and symptomatic responses
Undifferentiated carcinoma	21	8	6	4	3	1
Squamous cell carcinoma	14	—	—	2	12	4
Adenocarcinoma	8	—	1	1	6	2
Unclassified forms	5	—	1	—	4	2
Totals	48	8	8	7	25	9

The effectiveness of 1-methyl-1-nitrosourea therapy for patients with carcinoma of the lung was not correlated with the increase in daily dose of the agent (table 10). It should be mentioned that nine of 23 patients who received the drug and had a positive objective effect received a dose less than 10 mg/kg (8–9 mg/kg); ie, this effect could be achieved with comparatively small doses which were relatively harmless with respect to myelotoxicity (table 7). Only five of 23 patients had leukopenia and thrombocytopenia. The nadir of leukocytes was not less than 2000 cells/mm^3 and the nadir of platelets was not less than 75,000 platelets/mm^3. Hematopoiesis normalized within 3–14 days after the 1-methyl-1-nitrosourea treatment was stopped.

Results of 1-methyl-1-nitrosourea therapy for patients with Hodgkin's disease are given in table 11. In all patients the disease was in a generalized stage. Many had lesions of the peripheral and visceral lymphatic nodes and of the lung, spleen, liver, and skin, with general symptoms of the disease and changes in hematopoiesis. Fifteen of 18 patients had previously received radiation therapy and/or repeated courses of chemotherapy several times with different antitumor agents (cyclophosphamide, vinblastine, dopan) and cortical hormones. The disease had progressed significantly

TABLE 10.—Single and total doses of 1-methyl-1-nitrosourea for patients with carcinoma of the lung treated with and without effect

Results of treatment	No. of patients treated	Single dose (mg/kg) Range	Average	Total dose (mg/kg) Range	Average
Positive	23	3–28	10.9	50–222	99.1
None	25	6–29	12.5	39–175	93.4

TABLE 11.—Response of patients with Hodgkin's disease to 1-methyl-1-nitrosourea

Disease stage	No. of patients treated	No. of objective responses (grade) 3	2	1	0
IIIB	7	5	—	1	1
IVB	11	7	1	1	2
Totals	18	12	1	2	3

by the start of the 1-methyl-1-nitrosourea therapy.

The marked efficacy of the drug against Hodgkin's disease is shown in table 11. Objective positive responses were noted in 15 of 18 treated patients (83.3%); in 12 of these patients (66.7%) the responses were very good. The objective remissions in all cases were accompanied by considerable subjective and symptomatic improvement. Specific lesions of all types of lymphatic nodes and of various organs and skin were sensitive to 1-methyl-1-nitrosourea action. The duration of remission induced by the drug in Hodgkin's disease was 1–17 months and the median duration was 6.4 months. Two patients were still in remission at the time this paper was written (for 8 and 16 months respectively).

It should be emphasized that the 1-methyl-1-nitrosourea therapy was effective even for the eight patients previously treated with other cytostatic agents (cyclophosphamide, vinblastine, dopan) and radiotherapy. By the time of the 1-methyl-1-nitrosourea treatment these patients were resistant to any other kind of therapy.

Single and total doses of 1-methyl-1-nitrosourea given to patients with Hodgkin's disease were lower than those given to patients with other tumors, including carcinoma of the lung (table 12). Nevertheless, the remarkable improvement observed for most patients with Hodgkin's disease probably reflects their high sensitivity to the drug. Significant objective responses were noted in three patients with this disease in stage IVB who were treated with 1-methyl-1-nitrosourea in single daily doses of 6.6–7.0 mg/kg.

A myelotoxic effect of the drug was observed in five patients with Hodgkin's disease; all of these patients had been treated previously with radiation therapy or alkylating agents. For four patients the nadir of the leukocyte count was not less than 1700 cells/mm^3, and the nadir of the thrombocyte count was not less than 60,000 platelets/mm^3. Hematopoiesis normalized within 5–17 days after the 1-methyl-1-nitrosourea treatment was stopped. Unimproved pancytopenia developed in one patient.

TABLE 12.—Single and total doses of 1-methyl-1-nitrosourea for patients with Hodgkin's disease

Single dose (mg/kg)		Total dose (mg/kg)	
Range	Average	Range	Average
6.6–16.1	8.8	25.7–88.9	66.0

If patients were sensitive to 1-methyl-1-nitrosourea therapy, the positive antitumor response appeared immediately after one or two injections of the drug. The patients felt better, their pains became weaker, short-windedness and cough disappeared, temperatures became normal, and the compression syndrome quickly decreased. After the third or fourth infusion an objective decrease of the palpable lymphatic nodes could be observed. During further treatment all signs of the disease quickly disappeared, hematopoiesis became normal, and lesions of the peripheral lymphatic nodes were no longer observed. In the case of successful treatment, repeated roentgenographic findings showed a decrease and disappearance of atelectasis, specific lung lesions, and hydrothorax. Decrease of tumor nodes and bronchial stenosis was seen in tomograms.

As summarized in table 8, the effect of 1-methyl-1-nitrosourea was greater in undifferentiated carcinoma of the lung and Hodgkin's disease than in the other tumors studied. However, it should be mentioned that the drug was evaluated in many tumor types in single patients and the data available may not be representative for the individual tumor types.

Significant and prolonged objective effects were observed in a patient with lymphoreticulum cell sarcoma of the colon and in a patient with reticulosarcoma of the testes. In other cases improvement was neither remarkable nor of long duration.

DISCUSSION AND CONCLUSION

Experimental studies with 1-methyl-1-nitrosourea showed that after administration six times at a dose of 10–20 mg/kg or with a single dose of 100–120 mg/kg, the agent did not cause severe suppression of hematopoiesis and was well tolerated by animals. Pharmacologic investigation (23) showed that ten oral doses of the agent at a level of 25–50 mg/

kg/day resulted in severe hematopoietic depression (leukopenia, thrombocytopenia, and reticulocytopenia), the development of aplastic processes in the tissues of the hematopoietic system, and the development of atrophy of the germinal elements of the testes. Administration orally for 10 days at a dose of 10 mg/kg did not cause critical blood cell depression; observed hypoplasia of the thymus was reversible.

This experimental study has demonstrated that 1-methyl-1-nitrosourea has a remarkable effect against nine tumor systems in mice and rats. The drug is also known to be highly effective against such animal tumors as plasmacytoma of hamsters (2), Gerene carcinoma (3), Flexner-Jobling carcinoma, Krebs ascites carcinoma (4), and others.

The kinetic method applied to our investigation enabled us to quantitatively describe the 1-methyl-1-nitrosourea action and to compare different schedules of treatment.

Clinical studies showed that this drug exhibited antitumor effects against undifferentiated carcinoma of the lung and generalized forms of Hodgkin's disease. The objective responses were always accompanied by subjective and symptomatic improvement. The frequency and duration of objective remissions after 1-methyl-1-nitrosourea therapy were not less than those observed after treatment with most agents presently being used for undifferentiated carcinoma of the lung and generalized forms of Hodgkin's disease. In a number of cases, the drug was also effective in patients resistant to other drugs.

The drug treatment was accompanied by several direct toxic reactions (nausea, vomiting, and diarrhea in some patients) and moderate myelotoxicity. The toxicity was not more intensive than that of such widely used drugs as sarcolysin, 5-fluorouracil, etc.

The results of these investigations enable us to recommend 1-methyl-1-nitrosourea for the palliative treatment of patients with undifferentiated carcinoma of the lung and Hodgkin's disease when chemotherapy is indicated.

LIST OF COMPOUNDS

BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-

CCNU: NSC-79037; CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate

Dopan: NSC-44629; CAS reg. No. 520-09-2; uracil, 5-[bis(2-chloroethyl)amino]-6-methyl-

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU

1-Methyl-1-nitrosourea: NSC-23909; CAS reg. No. 684-93-5

Sarcolysin: NSC-14210; alanine, 3-[*p*-[bis(2-chloroethyl) amino]phenyl]-, monohydrochloride, DL-; phenylalanine mustard

Vinblastine: NSC-49842; CAS reg. No. 6449-03-2; vincaleukoblastine, sulfate (1:1), monohydrate

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Cytotoxicity of Antitumor Agents in a Synchronous Mammalian Cell System [1,2,3]

B. K. Bhuyan and T. J. Fraser [4]

SUMMARY

The cell-cycle phase specificity of several antitumor agents of clinical interest is reported. The order of sensitivity for cells in the different phases was as follows: (*a*) Alkylating agents (mechlorethamine, dimethane sulfonate): M, G_1, early S > mid-S > G_2. (*b*) Adriamycin: S > G_1, M, G_2. (*c*) Bleomycin: M > G_1 > S, G_2. (*d*) Mithramycin: late G_1, early S > mid-S, G_2 > early G_1, M. (*e*) Methyl-CCNU: late G_1 > M, G_1, G_2, S. (*f*) Diglycolaldehyde: late G_1, early S > M, early G_1, mid-S > G_2. (*g*) Busulfan: M = G_1 = S = G_2. Several compounds (procarbazine, isophosphamide, 5-methyl-THF, methotrexate, 6-methylmercaptopurine riboside, and guanazole) had such low cytotoxicity after 2-hour contact with cells that they could not be effectively evaluated in this system.

[Cancer Chemother Rep 58:149–155, 1974]

The variation in sensitivity of cultured mammalian cells through the division cycle to different agents has been reported by Mauro and Madoc-Jones (1), Bhuyan et al (2), and others (3,4). Such studies may be useful in designing combinations of drugs on a rational basis. Thus, two drugs affecting the same phase of the cell cycle, if given together, will not result in improved cell-kill, although they may delay the development of resistance to either drug.

We are reporting the cell-cycle phase specificity of several clinically active antitumor agents.

[1] Received Mar 20, 1973; revised Sept 12, 1973; accepted Sept 19, 1973.

[2] Supported in part by contract PH43-68-1023 from the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3] Drugs used in this study were supplied by Drug Research and Development, DCT, NCI. Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are included in the List of Compounds at the end of the text.

[4] Cancer Research, The Upjohn Company, Kalamazoo, Mich.

METHODS

DON cells, from a Chinese hamster fibroblast line (ATCC CCL 16), were grown at 37 C in McCoy's 5A medium modified by the addition of lactalbumin hydrolysate (0.5 g/liter) and Panmede (Paines and Pyrne Ltd, Greenford, England) (0.5 g/liter). The medium was obtained from the Grand Island Biological Company, Grand Island, New York. The cells were grown in 8-oz prescription bottles planted with about 2×10^6 cells in 25 ml of medium and were maintained in logarithmic growth by subculture every 2 days.

Synchronous DON cells were obtained by selectively harvesting mitotic cells. Cell survival was determined by plating and counting colonies after 7–9 days incubation as described previously (2). The plating efficiencies were about 40% for synchronous cells. For calculation of the percentage of survivals, the control (no drug) samples were normalized to 100% survival. The coefficient of variation in determining cell survival was about 15%, the coefficient of variation being the standard deviation expressed as a percentage of the mean.

The characteristics of the synchronous cultures (described previously, *see* ref 2) used in these experiments were determined at frequent intervals by three methods: (*a*) labeling the cells by exposure to a 30-minute pulse of ^{3}H-TdR (2 curies/mM, 10 microcuries/ml) and determining the percent of labeled cells, (*b*) determining the percent of cells killed by a short exposure to HSA-^{3}H-TdR[5] (20 curies/mM, 10 microcuries/ml) or to cytosine arabinoside (100 μg/ml), and (*c*) determining the mitotic index and the cell number. The results showed that mitosis was essentially completed within 1 hour after plating; the cells entered S at about 2 hours and stayed in S until about 8 hours. The second mitosis occurred about 11 hours after plating. As a typical example, the percentages of survival of cells exposed to HSA-^{3}H-TdR, from zero to 2 hours, 3 to 5 hours, and 8 to 10 hours, were 80.5, 18, and 77, respectively. This shows that in this experiment 80% of the cells from zero to 2 hours were in G_1, 82% of the cells from 3 to 5 hours were in S, and 77% of the cells from 8 to 10 hours were in G_2.

The solubilities of the drugs are as follows: procarbazine, isophosphamide, diglycolaldehyde, dimethane sulfonate, guanazole, 6-methylmercaptopurine riboside, mechlorethamine, adriamycin, and bleomycin were H_2O soluble. Methotrexate and 5-methyl-THF were dissolved in a small volume of 7.5% $NaHCO_3$ and diluted with saline. Mithramycin and busulfan were dissolved at 10 mg/ml in ethanol. Methyl-CCNU was dissolved at 10 mg/ml in DMSO. NSC-82196 was dissolved at 10 mg/ml in 0.01N HCl.

RESULTS

Dose-Response Studies

In order to estimate the concentration of drug to be used in phase-specificity studies, asynchronous DON cells were exposed to the drug for 2 hours and the percent survival was determined. Since the G_1 and G_2 phases of DON cells last about 2 hours (2), it was necessary to limit the exposure period to 2 hours in order to expose cells to the drug in these phases. The following agents were practically noncytotoxic (50%–100% survival) when asynchronous DON cells were exposed to the agents for 2 hours: procarbazine (200 μg/ml), isophosphamide (200 μg/ml), 5-methyl-THF (500 μg/ml), guanazole (500 μg/ml), 6-methylmercaptopurine riboside (500 μg/ml), and methotrexate (500 μg/ml).

Typical dose-response curves obtained with adriamycin, mechlorethamine, mithramycin, and dimethane sulfonate are shown in figure 1. The percent survival obtained after exposure of cells to the following drugs for 2 hours was: methyl-CCNU (4 μg/ml), 14.8%; busulfan (60 μg/ml), 50%; diglycolaldehyde (100 μg/ml), 95%; and NSC-82196 (100 μg/ml), 100%.

Procarbazine, Isophosphamide, 5-Methyl-THF, Guanazole, 6-Methylmercaptopurine Riboside, and Methotrexate

Since G_1 and G_2 cells each constitute only 15% of the total cell number in an asyn-

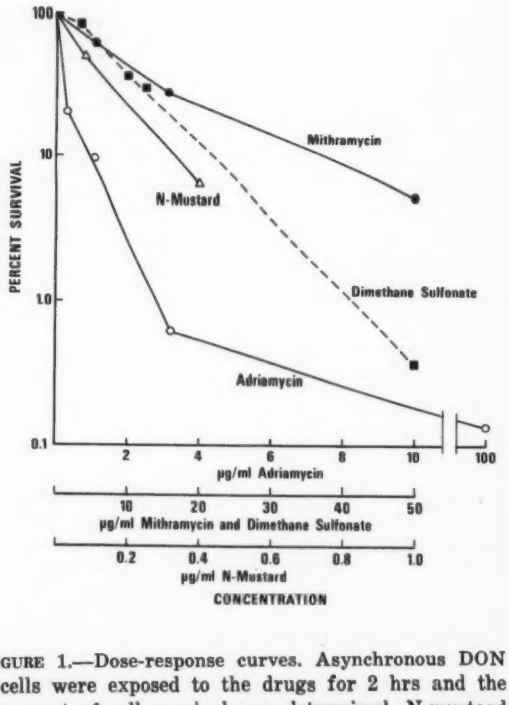

FIGURE 1.—Dose-response curves. Asynchronous DON cells were exposed to the drugs for 2 hrs and the percent of cell survival was determined. N-mustard = mechlorethamine.

[5] HSA-^{3}H-TdR = high specific activity thymidine-^{3}H (20 curies/mM).

chronous population, complete cell-kill of the G_1 and G_2 cells would result in only 15% of the total population being killed. Therefore, synchronous cells were exposed to high levels of the drug and the results are shown in table 1. The results clearly indicate that none of these agents were specifically cytotoxic to any particular phase of the cell cycle except for the slight cytotoxicity of 6-methylmercaptopurine riboside and isophosphamide to M–G_1 cells.

Response to Alkylating Agents

Mechlorethamine, an alkylating agent, is the drug of choice in the treatment of advanced Hodgkin's disease and is used to treat patients with lymphosarcoma and well-differentiated carcinoma of the lung (5). Our results (fig 2) show that cells in mitosis, G_1, and early S are most sensitive to the drug. Cells in mid-S and G_2 are relatively insensitive to mechlorethamine. These results are in accord with those reported by Mauro and Madoc-Jones (1) and Walker and Helleiner (6).

NSC-82196 is an alkylating agent which was found to be active when administered orally against L1210 leukemia and several other animal tumors (7,8). It is presently undergoing clinical trial (9). Our results (fig 2) indicate that the difference in the sensitivity of the cells in different phases of the cell cycle was not as great as that seen with mechlorethamine. NSC-82196 was also much less cytotoxic than mechlorethamine or dimethane sulfonate since 500 μg/ml of NSC-82196 killed only about 20% of the cells as compared to about 80% cells killed by 0.2 μg/ml of mechlorethamine.

Dimethane sulfonate is an alkylating agent effective in the treatment of chronic myelogenous leukemia (10). This drug, like mechlorethamine, was most cytotoxic to cells in M and G_1. The cells became less sensitive to the agent as they progressed through S and G_2 (fig 2).

Busulfan also acts as an alkylating agent and is effective in the treatment of chronic myelogenous leukemia (5). This drug was of low cytotoxicity and lacked any phase specificity (fig 2).

Adriamycin, Mithramycin, and Bleomycin

The antibiotics adriamycin, bleomycin, and mithramycin are considered together. Adriamycin is effective in the treatment of several experimental tumors. In clinical trials the drug caused consistent regressions in ten histologically different types of tumors but was most active against acute lymphoblastic and chronic (myeloid and lymphocytic) leukemias (11). The results (fig 3) show that adriamycin was most cytotoxic to cells in S while cells in G_1 and G_2 were much less sensitive to the drug. At high concentrations, however, substantial proportions of the cells in G_1 and G_2 were killed.

Bleomycin is clinically active against several well-differentiated squamous cell carcinomas (12). It markedly inhibits DNA synthesis with

TABLE 1.—Survival of synchronous cells exposed to different agents*

	% Survival		
	M–G_1 phases	Mid-S phase	S–G_2 phases
Procarbazine	90	100	95
Isophosphamide	68	96	103
5-Methyl-THF	98	100	97
Methotrexate	95	112	104
Guanazole	96	87	88
6-Methylmercaptopurine riboside	77	90	98

*Mitotic cells were plated and the cells were exposed to the agents for 2 hrs in different phases of the cell cycle. All agents were used at 500 μg/ml.

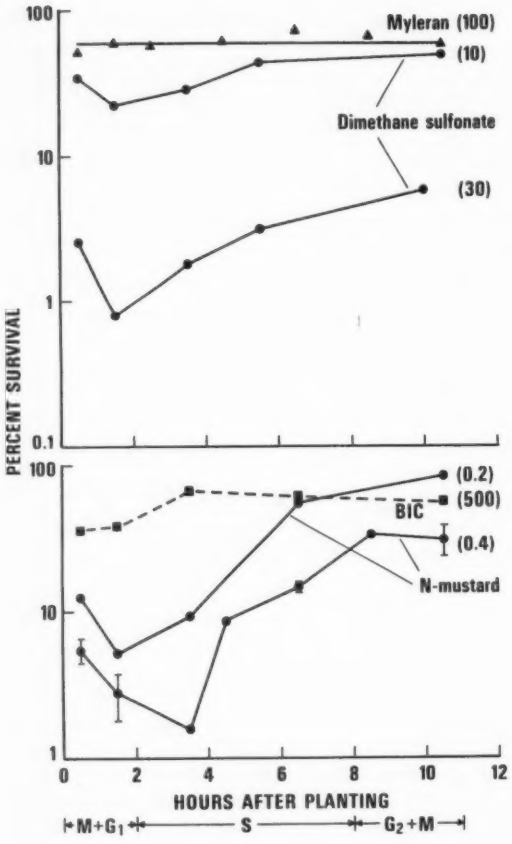

FIGURE 2.—Sensitivity of synchronous DON cells to alkylating agents. Mitotic cells were exposed to the drugs for 1-hr periods after plating. The drugs were removed after 1 hr, and the cells were diluted and plated (12 plates for each sample). Colonies were counted after about 7 days' incubation. The times shown are half-way points in the period of exposure, ie, cells exposed at 1 hr after plating and harvested at 2 hrs are shown at 1½ hr. In these experiments, the ½-hr point indicates cells in M and G_1; the 1½-hr point indicates cells in late G_1 or G_1–S; the 3½- and 6½-hr points indicate cells in S; and the 10½-hr point indicates cells in G_2 and M mixed with a certain percent of cells in S. The numbers in parentheses indicate drug concentration in μg/ml. The bars (for mechlorethamine [N-mustard]) indicate the average deviation between two different experiments. BIC = NSC-82196; Myleran = busulfan.

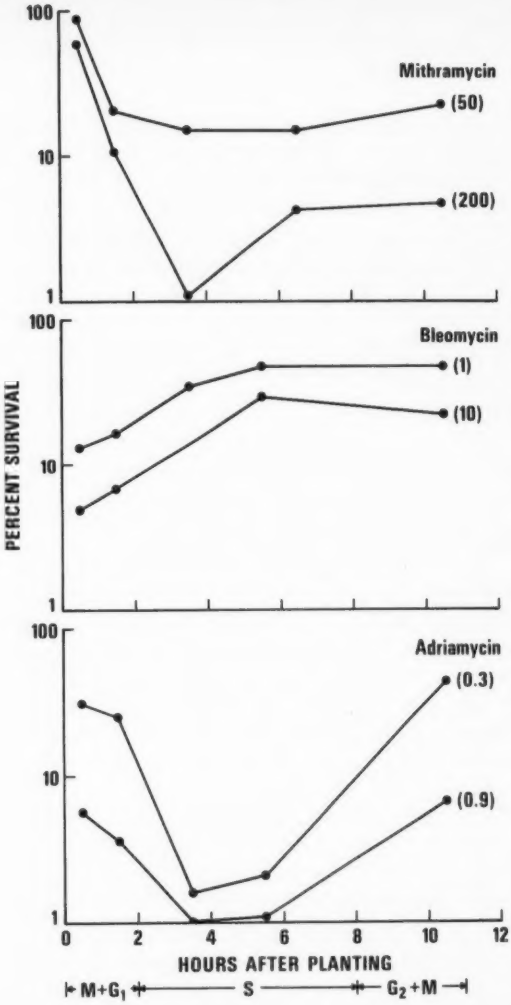

FIGURE 3.—Sensitivity of synchronous DON cells to adriamycin, bleomycin, and mithramycin; protocol same as for fig 2.

much less inhibition of RNA or protein syntheses (13). The results (fig 3) show that mitotic and G_1 cells were most sensitive to the drug while S and G_2 cells were much less sensitive.

Mithramycin is active in the clinic in embryonal cell carcinoma of the testis, hypernephroma, and glioblastoma multiforme (14). It binds to DNA and markedly inhibits RNA synthesis with little or no inhibition of protein synthesis (15). Figure 3 shows that mithramycin was least cytotoxic to cells in M and G_1 while cells in S and G_2 were sensitive to the drug.

Methyl-CCNU and Diglycolaldehyde

Methyl-CCNU is highly active in the treatment of advanced lung carcinoma in the mouse and is being tested in the clinic (16). Like CCNU (2), methyl-CCNU was most active against cells in late G_1 or G_1–S transition (fig 4).

Diglycolaldehyde was most active against cells in late G_1 or early S (fig 4).

DISCUSSION

The reason for the low cytotoxicity of several of the compounds is not completely clear. Isophosphamide, an analog of cyclophosphamide, will probably need to be activated in vivo in order to exert its cytotoxicity. A compound such as methotrexate probably kills cells in S phase by inhibiting DNA synthesis. However, methotrexate needed about 10 hours of contact with L-cells in order to exert its cytotoxic effect (17). A similar consideration might also apply to 6-methylmercaptopurine riboside, 5-methyl-THF, and others.

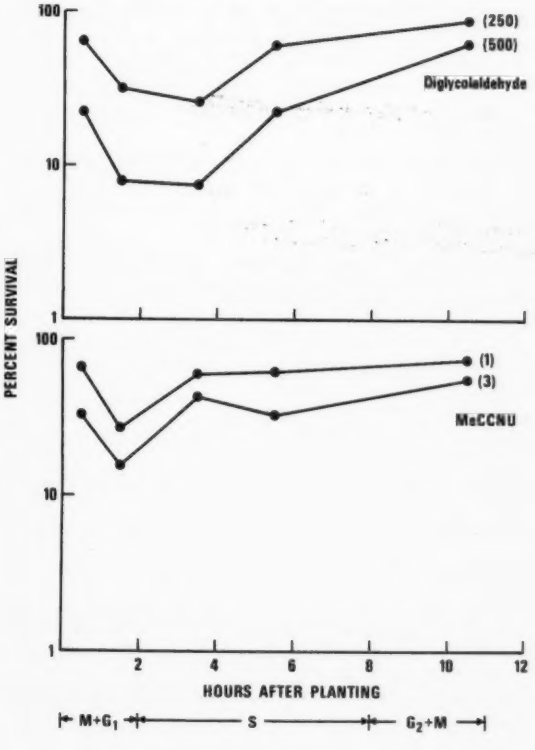

FIGURE 4.—Sensitivity of synchronous DON cells to methyl-CCNU (MeCCNU) and diglycolaldehyde; protocol same as for fig 2.

The alkylating agents reported here (mechlorethamine, dimethane sulfonate, but not busulfan) and those reported previously (chlorambucil, thioTEPA, and melphalan [2]) were all characterized by toxicity to cells in M, G_1, or G_1–S transition. Similar results were reported for sulfur mustard, uracil mustard, dimethyl myleran, ethyl methane sulfonate, and others by Mauro and Madoc-Jones (1). This common site for several alkylating agents may indicate vulnerability of mitotic chromosomes or DNA before replication.

Adriamycin (an analog of daunorubicin) was shown to be most active against cells in S. Similar results with adriamycin were reported by Kim and Kim (18) using synchronized HeLa cells. Tobey (19) reported that both daunorubicin and adriamycin did not grossly inhibit the transition of G_1 cells into S but were extremely effective in blocking the cells from reaching mitosis.

The results obtained with bleomycin by Barranco and Humphrey (3), by Terasima and Umezawa (20), and by us differ from each other. Thus, for Chinese hamster ovary cells (3) the order of sensitivity was M > G_2 > early S > late S > G_1, whereas with DON cells (this paper) the order was M > G_1 > early S > mid-S > G_2. With HeLa S_3 cells (20), the order was M > early S > late S > G_2, G_1. All of our results are in agreement in finding that M cells were most sensitive while the S-phase cells were moderately sensitive to the drug. It is not known whether the differences in cell lines or the differences in the synchronization procedure account for the observed differences in phase sensitivity.

Mithramycin primarily affects RNA synthesis with very little effect upon DNA synthesis (15). Tobey reported the similarity of the effects of mithramycin (19) and actinomycin D on the progression of CHO cells through the cell cycle. Thus, with both agents the entry of cells into S was hardly affected, while completion of G_2 was inhibited. In our cell survival experiments (fig 3), the cells in G_1–S transition were most sensitive to the drug, in a manner

similar to that shown with actinomycin D (2). With actinomycin D, however, the cells in S were much more sensitive than the cells in G_2, while with mithramycin the cells in G_2 and mid-S were equally sensitive.

Our studies suggest several combinations that might result in improved killing of the cells. Bleomycin (G_1 sensitive) could be combined with adriamycin (S sensitive) and mithramycin (S–G_2 sensitive), or the alkylating agents (M–G_1 sensitive) could be combined with mithramycin. However, the efficacy of combinations will also depend on the phase of the cell cycle where cells accumulate due to the effect of the drug.

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LIST OF COMPOUNDS

Actinomycin D: NSC-3053
Adriamycin: NSC-123127; 14-hydroxy-daunomycin
Bleomycin: NSC-125066
Busulfan: NSC-750; CAS reg. No. 55-98-1; 1,4-butanediol, dimethanesulfonate; Myleran
CCNU: NSC-79037; CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-
Chlorambucil: NSC-3088; CAS reg. No. 305-03-3; butyric acid, 4-[*p*-[bis(2-chloroethyl)amino]phenyl]-
Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate
Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride
Daunorubicin: NSC-83142; CAS reg. No. 23541-50-6
Diglycolaldehyde: NSC-118994; diglycolaldehyde, α-(hydroxymethyl)-α′-(6-hydroxy-9*H*-purin-9-yl)-
Dimethane sulfonate: NSC-102627; CAS reg. No. 3458-

22-8; 1-propanol, 3,3'-iminodi-, dimethanesulfonate (ester), hydrochloride

Dimethyl myleran: NSC-23890; CAS reg. No. 55-93-6; 2,5-hexanediol, dimethanesulfonate

Ethyl methane sulfonate: NSC-26805; CAS reg. No. 62-50-0; methanesulfonic acid, ethyl ester

Guanazole: NSC-1895; CAS reg. No. 503-88-8; 1,2,4-triazolidine, 3,5-diimino-

Isophosphamide: NSC-109724; CAS reg. No. 3778-73-2; 1,3,2-oxazaphosphorine, 3-(2-chloroethyl)-2-[(2-chloroethyl)amino]tetrahydro-, 2-oxide

Mechlorethamine: NSC-762; CAS reg. No. 55-86-7; diethylamine, 2,2'-dichloro-*N*-methyl-, hydrochloride; nitrogen mustard

Melphalan: NSC-8806; CAS reg. No. 3223-07-2; alanine, 3-[*p*-bis(2-chloroethyl)amino]phenyl]-, monohydrochloride, L-; L-phenylalanine mustard

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)-methyl]methylamino]benzoyl]-

Methyl-CCNU: NSC-95441; CAS reg. No. 33073-59-5; urea, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitroso-

6-Methylmercaptopurine riboside: NSC-40774; CAS reg. No. 7143-67-1; 9*H*-purine, 6-(methylthio)-9-β-D-ribofuranosyl-, dihydrate

5-Methyl-THF: NSC-139490; glutamic acid, *N*-[*p*-[[2-(2-amino-5,6,7,8-tetrahydro-4-hydroxy-5-methyl-6-pteridinyl)ethyl]amino]benzoyl]-, L-

Mithramycin: NSC-24559; CAS reg. No. 18378-89-7

NSC-82196: CAS reg. No. 5034-77-5; imidazole-4-carboxamide, 5-[3,3-bis(2-chloroethyl)-1-triazeno]-; BIC

Procarbazine: NSC-77213; CAS reg. No. 491-88-3; *p*-toluamide, *N*-isopropyl-α-(2-methylhydrazino)-, monohydrochloride

Sulfur mustard: CAS reg. No. 505-60-2; sulfide, bis(2-chloroethyl)-

ThioTEPA: NSC-6396; CAS reg. No. 52-24-4; phosphine sulfide, tris(1-aziridinyl)-

Uracil mustard: NSC-34462; CAS reg. No. 66-75-1; uracil, 5-[bis(2-chloroethyl)amino]-

Tissue Distribution of Streptozotocin (NSC-85998) [1,2]

B. K. Bhuyan, S. L. Kuentzel, L. G. Gray, T. J. Fraser, D. Wallach, and G. L. Neil [3]

SUMMARY

The streptozotocin level in tissues was determined by antibacterial assay, chemical assay (measures *N*-nitroso group of streptozotocin), and radiochemical assay. On the basis of the antibacterial and chemical assays, streptozotocin levels in plasma decreased rapidly with a half-time of 10–15 minutes. Radiochemical assay of plasma gave consistently higher values, indicating extensive degradation of the drug. Streptozotocin levels were maintained longer in the tissues than in the plasma. Thus, 1 hour after administration, only 10 μg/ml streptozotocin was present in the plasma as compared to 90 μg/g in the liver. The highest concentration of streptozotocin was found in the liver, and the next highest was in the kidney. The chemical and radiochemical assays for streptozotocin in all tissues gave higher values than the antibacterial assay. Streptozotocin was so rapidly excreted through the urine that 72% of the total ^{3}H-streptozotocin injected could be accounted for in the urine 4 hours later. Radiochromatograms of plasma, liver, and urine extracts showed the presence of five radioactive components. Two of these components had antibacterial activity and were most likely the α- and β-anomers of streptozotocin.

[Cancer Chemother Rep 58:157–165, 1974]

Streptozotocin is an antibiotic with broad spectrum antibacterial, antitumor, mutagenic, and diabetogenic activities (1–3). The diabetogenicity of streptozotocin is due to its selective toxicity for pancreatic beta cells (4). This selective toxicity was useful in the treatment of patients with metastatic insulinoma (5). Streptozotocin is the 2-deoxy-D-glucose derivative of 1-methyl-1-nitrosourea (6)[4] and has been shown to inhibit primarily DNA synthesis in bacterial and mammalian cells (7,8). The present study reports streptozotocin levels in the blood and tissues of several animal species.

MATERIALS AND METHODS

Streptozotocin Assays

Streptozotocin can be assayed by bioassay using *Proteus rettgeri* as the test organism (9), by chemical assay measuring the *N*-nitroso group (10), or by radiochemical assay.

Antibacterial assay.—*P. rettgeri* was more sensitive than the following organisms tested: *Lactobacillus casei, Klebsiella pneumoniae, Salmonella pullorum, Staphylococcus aureus, Escherichia coli, Bacillus subtilis, Sarcina lutea,* and *P. vulgaris.* The disc-plate method of assay (using *P. rettgeri* as the test organism)

[1] Received Apr 13, 1973; revised Oct 18, 1973; accepted Oct 25, 1973.

[2] Supported in part by contract PH43-68-1023 from the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

[3] Research Laboratories, The Upjohn Company, Kalamazoo, Mich.

[4] 1-Methyl-1-nitrosourea: NSC-23909; CAS reg. No. 684-93-5.

described by Sokolski et al (9) was employed in these studies. The assay could detect 1 μg/ml streptozotocin. The sensitivity of the assay could be increased twofold by incubating the *P. rettgeri* test plates after application of antibiotic-containing discs for 4 hours in the refrigerator prior to incubation at 32 C. The stability of streptozotocin in different diluents is shown in table 1. Streptozotocin was unstable in plasma alone and in plasma acidified with hydrochloric acid (HCl) (9). Streptozotocin was stable in phosphate buffer (0.1 M potassium phosphate, pH 4), in acetate buffer (0.1 M acetic acid, 0.02 M sodium acetate, pH 4), in mouse plasma diluted at least threefold with phosphate buffer, or in liver homogenate prepared in phosphate buffer. Therefore, immediately after collection, all tissue samples were diluted with buffer and stored in ice. The effect of different diluents on the streptozotocin standard curve (zone vs log concentration) is shown in figure 1. Streptozotocin solutions prepared in tissue homogenate or plasma diluted threefold with phosphate buffer yielded standard curves not significantly different from that obtained with streptozotocin in phosphate buffer alone. The standard curve with plasma as diluent was significantly different from that obtained with phosphate buffer as diluent. Therefore, all tissue and biologic fluids were stored in ice after homogenization in or dilution (at least threefold) with phosphate buffer prior to bioassay, and the zone sizes were compared to a standard curve obtained with streptozotocin diluted in phosphate buffer. We attempted to dilute all samples so that they contained about 3–10 μg/ml streptozotocin, enabling us to use the standard curve with phosphate buffer. Streptozotocin levels determined by antibacterial assay are referred to as "streptozotocin bioequivalents."

Chemical assay.—The assay method described by Forist (10) was employed. This method depends on the cleavage of the *N*-nitroso group of streptozotocin to yield nitrous acid. This nitrous acid is diazotized with sulfanilic acid and the diazo compound is coupled with *N*-(1-napthyl)-ethylene-diamine to produce a colored azo compound with an absorption maximum at 550 mμ. Portions of samples (obtained by pretreatment of tissues as described below) were diluted to 1 ml with H_2O. To this was added 1 ml of 6N HCl and 5 ml of color reagent (*see* below). The solutions were mixed and heated in a boiling H_2O bath for 5 minutes, after which the tubes were cooled to room temperature and the optical density was determined at 550 mμ. Color reagent was prepared by dissolving *N*-(1-napthyl)-ethylene-diamine dihydrochloride (2 mg/ml) in a solution of 1% sulfanilic acid in 30% acetic acid. The resulting solution was diluted 1:1 with 30% acetic acid prior to use. In the chemical assay, the following diluents gave essentially the same results: acetate buffer (*see* under *Antibacterial assay*), 5% perchloric acid (PCA), or the protein-free supernatants of PCA-treated tissue homogenates. Streptozotocin levels determined by chemical assay are referred to as "streptozotocin chemical equivalents."

Radiochemical assay.—Mice were injected intraperitoneally (ip) with 950 microcuries

TABLE 1.—Stability of streptozotocin in different diluents

Incubation (hrs)	Streptozotocin remaining (%)								
	Phosphate*			Plasma			Plasma-phosphate†		
	-4 C	4 C	25 C	-4 C	4 C	25 C	-4 C	4 C	25 C
2	92	97	92	70	58	12	100	100	100
6	100	100	94	79	63	0	80	100	100
24	100	100	100	46	29	0	100	92	44

*The phosphate buffer was 0.1 M potassium phosphate adjusted to pH 4. Streptozotocin (100 μg/ml) was incubated in different diluents at the temperatures indicated. Samples were taken at different times, diluted in phosphate buffer, and bioassayed.

†The plasma-phosphate consisted of 1 part mouse plasma plus 2 parts phosphate buffer.

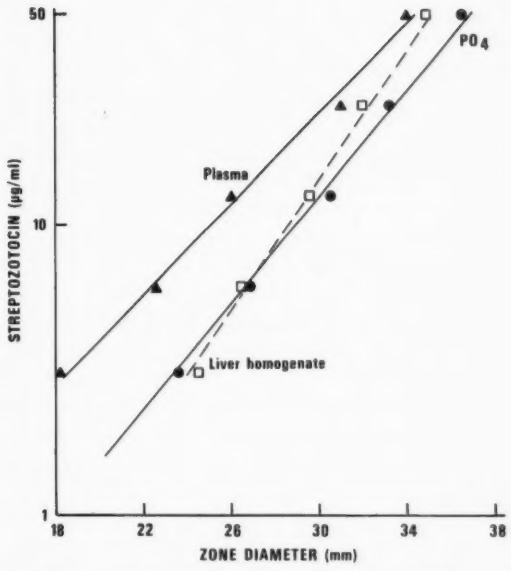

FIGURE 1.—Bioassay of streptozotocin in different diluents. Streptozotocin was diluted in mouse plasma alone (▲), mouse liver homogenate diluted threefold with 0.1 M potassium phosphate (pH 4) (□), or potassium phosphate alone (●). The solutions were applied (0.08 ml) to 13.5-mm paper discs and the discs placed on *P. rettgeri* plates. Zones of inhibition were measured after overnight incubation at 32 C.

(296 mg) streptozotocin/kg of body weight. Radioactivity of samples of plasma, urine, fecal extracts (with water), and tissue homogenate supernatants was determined by direct scintillation counting in Diotol (11).[5] Samples of plasma and urine were distilled and the tritiated water content was determined.[6] Results were expressed as μg streptozotocin radioequivalents/ml of fluid or radioequivalents/g of wet weight tissue. Radiochromatography was carried out using cellulose thin-layer chromatography (TLC) plates (Brinkmann Instruments, Inc, Westbury, NY) and a solvent system consisting of 85% acetone:15% H_2O. The TLC plates were developed by ascending chromatography for 2 hours. Streptozotocin standards were visualized under ultraviolet light. Chromatography of apparently pure streptozotocin (either unlabeled or ^{3}H-labeled) in the solvent system just described gave two components with R_f values of 0.63 and 0.75, respectively. These two compounds were at least partially interconvertible as shown by two-dimensional chromatography. Both compounds were bioactive against *P. rettgeri*. These components are probably the α- and β-anomers of streptozotocin.

[5] All counts were corrected for quenching by adding ^{3}H-toluene and recounting.

[6] The tritiated water content varied with time (range, 0.1%–71%) and appropriate corrections were made.

Disposition Studies in Mice

Streptozotocin (tritiated or unlabeled) was dissolved in phosphate buffer and was administered ip to 20-g female BDF_1 (C57BL/6 × DBA/2) mice (Jackson Memorial Laboratory, Bar Harbor, Me). At various times thereafter, groups of mice (four or five per group) were anesthetized with Metofane (Pitman-Moore, Inc, Washington Crossing, NJ); heparinized blood was obtained from the vena cava and tissues were excised.

(*a*) *Plasma assay.*—Blood from each group was pooled and plasma was decanted after centrifugation (5000 *g*, 5 min, 4 C). Samples of plasma were diluted with at least two volumes of ice-cold phosphate buffer for bioassay or with one volume of cold 5% PCA for chemical assay. After centrifugation, the deproteinized supernatant was used for chemical assay. Radioactivity in plasma was determined directly by the scintillation counting technique. For radiochromatography, plasma (obtained 30 min after administering ^{3}H-streptozotocin) was deproteinized with two volumes of ethanol.

(*b*) *Tissue assay.*—Tissues were excised, rinsed in ice-cold phosphate buffer, pooled, weighed, and homogenized in three volumes of ice-cold phosphate buffer. The homogenate was centrifuged at 5000 *g* for 10 minutes at 4 C and the supernatant was used for chemical assay and bioassay. For bioassay the supernatant was diluted with phosphate buffer. For chemical assay the supernatant was treated with equal volumes of cold 5% PCA and centrifuged. The protein-free supernatant was diluted with acetate buffer for chemical assay. Radioactivity of homogenate supernatants was determined directly (where appropriate) using a Diotol counting solution. The supernatant of liver homogenate obtained 2 hours after the

injection of ^{3}H-streptozotocin was lyophilized and reconstituted in a small volume of buffer for radiochromatography.

(*c*) *Urine and fecal assay.*—Three groups of mice treated with ^{3}H-streptozotocin were housed in metabolism cages (Acme Natural Products, Chicago, Ill) and urine and feces were collected 4, 12, and 24 hours after drug administration. Radioactivity in the feces was determined. The urine collected over the first 4 hours after ^{3}H-streptozotocin administration was radiochromatographed.

Recovery and Reproducibility

When streptozotocin was added to blood or liver homogenate, about 98% of the total streptozotocin could be recovered by both chemical assay and bioassay.

To determine the variation between mice, five mice were injected ip with 100 mg/kg of streptozotocin. Plasma and livers from individual mice were assayed. The coefficients of variations (standard deviation expressed as the percent of the mean) were five and 19 for chemical assay and bioassay, respectively. Therefore, in all subsequent experiments, five mice were used and the tissue samples were pooled and assayed.

Studies With Rats, Cats, Dogs, and Monkeys

Streptozotocin was injected intravenously (iv); the tissue samples were treated as described above, and streptozotocin equivalents were determined by chemical assay.

Materials.—^{3}H-streptozotocin was prepared according to the method of Hessler and Jahnke (12) by the Monsanto Research Corporation. The tritiated material had a specific activity of 37.565 millicuries/mM and the ^{3}H was present on carbon–6 of the glucose moiety.

RESULTS

Plasma Level Studies

Streptozotocin equivalents in mouse plasma were determined by radioactivity measurements or by chemical and biologic assays after ip administration of 300 mg/kg of ^{3}H-streptozotocin. These results and the streptozotocin equivalents obtained by chemical assay and bioassay after ip doses of 100 and 1000 mg/kg of nonradioactive streptozotocin are shown in figure 2. The results indicate excellent agreement between the chemical assay and bioassay values at all doses. Streptozotocin bioactivity or chemical activity disappeared from mouse plasma with an initial half-life of about 10–15 minutes. The streptozotocin bioequivalents and chemical equivalents were lower than the streptozotocin radioequivalents, indicating extensive metabolism of the drug. Thus, 1 hour after administration, only about 20% of the tritiated material in the plasma was bioactive or gave a positive chemical test. Twenty-four hours after administration, 8-μg/ml streptozotocin radioequivalents were present as compared to undetectable (ie, < 1 μg/ml) streptozotocin bioequivalents or chemical equivalents.

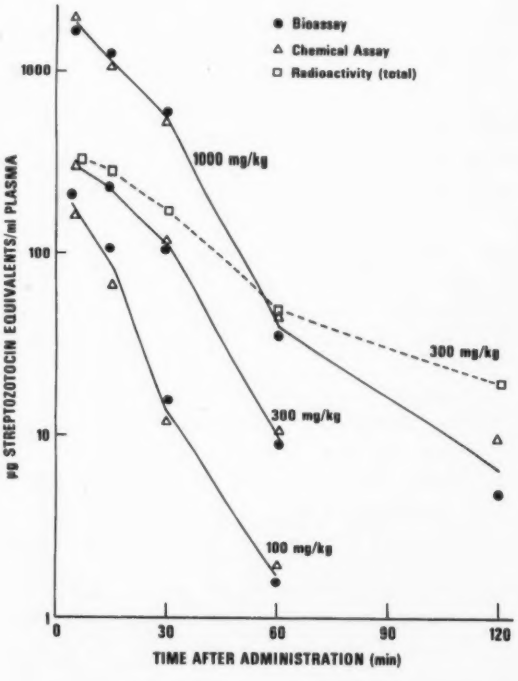

FIGURE 2.—Streptozotocin equivalents in mouse plasma after ip administration of 100, 300, or 1000 mg/kg streptozotocin. Streptozotocin levels were determined by either bioassay, chemical assay, or total radioactivity measurements. The details of the assay are outlined under Materials and Methods.

Streptozotocin chemical equivalents in the plasma of dogs treated iv or intramuscularly (im) with 20 mg/kg of the drug are shown in figure 3. After iv injection, streptozotocin chemical equivalents disappeared from the plasma with an initial half-life of about 4–5 minutes. Maximum plasma levels after im injection were eight times lower than those obtained after iv injection.

Streptozotocin Levels in Tissues

Levels of streptozotocin equivalents in tissue homogenates as determined by radioactivity assay, chemical assay, or bioassay are compared for the liver, kidney, and pancreas in table 2. In all cases streptozotocin radioequivalents were greater than those obtained by chemical assay, while the bioassay gave the lowest values of all. For example, less than 20% of the radioequivalents in the liver were bioactive. The disposition of radioactivity in various tissues of mice after ip administration of ^{3}H-streptozotocin (300 mg/kg) is illustrated in figure 4. Results are expressed as μg streptozotocin radioequivalents/g of tissue. Three tissues (liver, intestine, and kidney) consistently appear to have greater concentrations of radioactivity than that observed in the plasma. Levels in the pancreas and lung at later times are also somewhat greater than those found in the plasma. By 24 hours, levels in all tissues are less than about 10 μg streptozotocin radioequivalents/g of tissue, values consistent with the rapid urinary excretion of radioactivity (*see* below). The high levels observed in the kidney at early times undoubtedly reflect this rapid renal clearance of radioactivity. Radioactivity levels in the brain were insignificant.

Streptozotocin chemical equivalents in the tissues of different animal species 30 minutes after iv injection are given in table 3. The results indicate that in all species, maximum accumulation of streptozotocin equivalents was seen in the liver and the kidney. In the monkey and maybe the rat, the drug accumulated more in the kidney than in the liver.

Pretreatment with nicotinamide protects animals from the diabetogenic effects of streptozotocin (13). Streptozotocin chemical equivalents and bioequivalents were determined in mouse tissues at various times after administration of a combination of streptozotocin (300 mg/kg) and nicotinamide (300 mg/kg), or streptozotocin (300 mg/kg) alone. Similar streptozotocin levels were obtained in the tissues (plasma, liver, kidney, and pancreas) when the combination or the drug alone was injected.

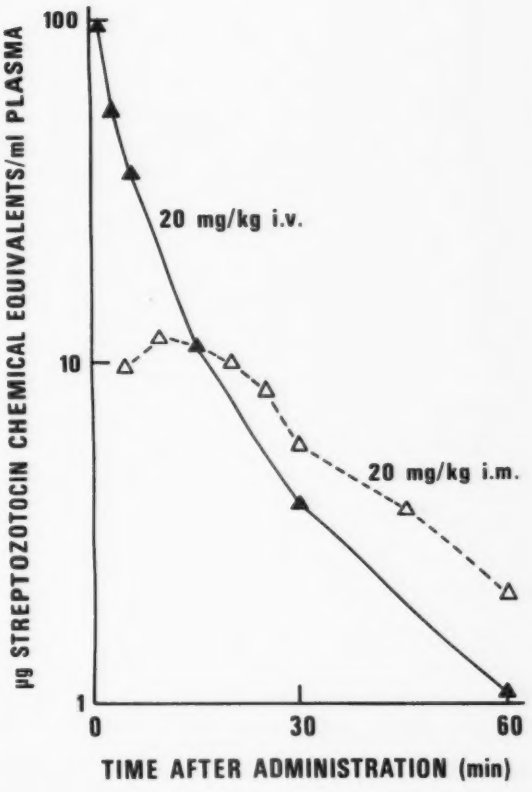

FIGURE 3.—Streptozotocin chemical equivalents in dog plasma. Dogs received 20 mg/kg streptozotocin iv or im. Plasma samples were taken at different times and streptozotocin levels were determined by chemical assay.

Excretion Studies

When ^{3}H-streptozotocin was administered ip to mice, 74% and 83% of the radioactivity was excreted in the urine by 4 and 24 hours after injection, respectively. A small amount of radioactivity was present in the fecal samples (about 2%–4% of that found in urine) and probably represents contamination by the urine.

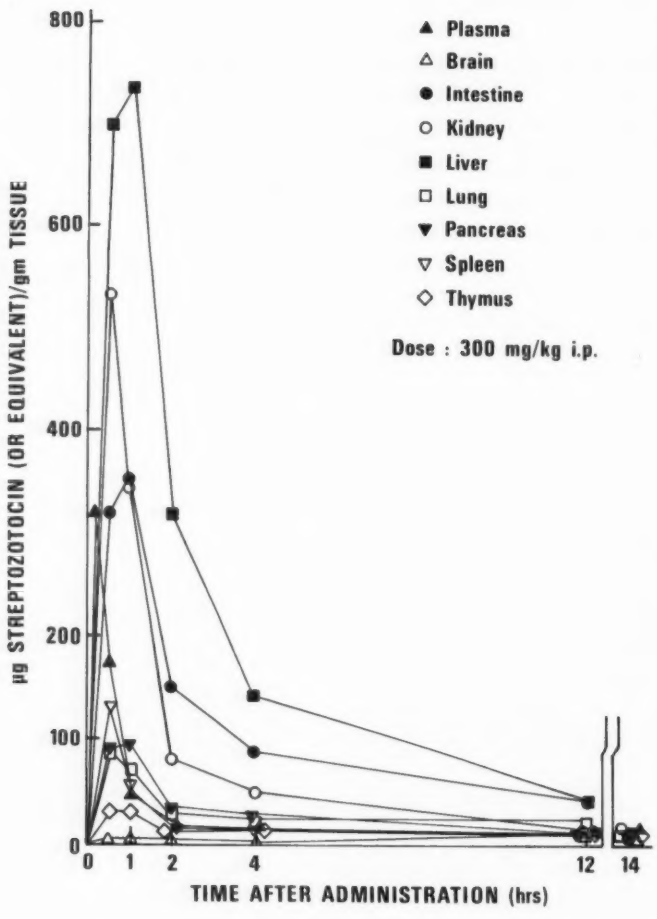

FIGURE 4.—Streptozotocin radioequivalents in mouse tissues after ip administration of 300 mg/kg ^{3}H-streptozotocin. The details of total radioactivity determination are given under Materials and Methods.

TABLE 2.—Comparison of streptozotocin equivalents in mouse tissue after ip administration of ^{3}H-streptozotocin

Tissue	Time (min)	µg Streptozotocin (equivalents)/g (tissue)*		
		^{3}H assay	Chemical assay	Bioassay
Liver	30	699	630	110
	60	739	510	91
	120	318	220	33
Kidney	30	533	340	146
	60	345	208	52
	120	80	ND†	16
Pancreas	30	90	81	33
	60	95	64	33
	120	31	ND†	ND†

*^{3}H-streptozotocin (300 mg/kg) was injected ip and streptozotocin levels were determined at different times by chemical assay, bioassay, and radioactivity measurement.

†ND = not determined.

TABLE 3.—Streptozotocin chemical equivalents in various tissues

Tissue	*Species:* *Dose (mg/kg):*	Mouse (100)	Rat (100)	Cat (30)	Monkey (100)	Dog (20)
		*Streptozotocin equivalents (μg/g wet weight tissue)**				
Liver		174.0	402.0	129.6	420.0	213.0
Kidney		67.5	489.0	141.6	1044.0	63.6
Heart		15.12	14.4	8.4	26.1	3.6
Lungs		5.1	26.7	15.6	56.1	7.5
Spleen		4.2	13.2	8.7	46.8	2.7
Smooth muscle		33.6	91.5	21.0	Not done	11.4
Skeletal muscle		6.9	8.7	4.8	75.9	2.7
Serum		13.2	13.6	20.2	0.8	5.0

*Streptozotocin was administered iv; drug levels in tissue were determined 30 min later by chemical assay. For this experiment mongrel dogs and cats, African green monkeys, Sprague-Dawley rats, and ICR mice were used.

Chromatographic Separation of Streptozotocin Metabolites

The composition of plasma, urine, and liver homogenate in mice treated ip with ^{3}H-streptozotocin (300 mg/kg) was determined by radiochromatography. Plasma and liver homogenates were obtained 30 minutes after administration, while the urine was collected over a 4-hour period. The results are shown in table 4. At least five radioactive components were detected in both the plasma and urine. Components IV and V (R_f values of 0.63 and 0.75, respectively) appear to represent the two anomers of streptozotocin itself. Together, these two components accounted for 49.8% and 50.6% of the total radioactivity in plasma and urine, respectively. However, less than 10% of the radioactivity in the liver homogenate supernatant had a chromatographic mobility characteristic of either streptozotocin anomer. A component of very low mobility (component I, R_f = 0.08) accounts for essentially all radioactivity in the liver homogenate. Components I, II, and III have not yet been identified.

When the radiochromatographic data are used to calculate the percent of streptozotocin present in the total radioactivity, the resulting streptozotocin levels (table 5) in plasma, urine, and liver homogenates agree very well with those determined by bioassay. For plasma and urine the chemical assay is also in good agreement.

However, as noted before with the liver homogenate (*see* table 2), the chemical assay yields much higher values than those obtained by bioassay or radiochromatography. Bioautography (with *P. rettgeri* as the test organism) of the urine of mice treated with ^{3}H-streptozotocin gave zones of inhibition of R_f values (0.63 and 0.75) corresponding to the two streptozotocin anomers.

DISCUSSION

Streptozotocin bioequivalents in plasma (determined by bioassay) decreased rapidly with an initial half-life of 10 minutes. Streptozotocin was also excreted rapidly in the urine such that within 4 hours, 72% of the drug could be found in the urine.

TABLE 4.—Radiochromatographic determination of the composition of plasma, urine, and liver homogenate after administration of ^{3}H-streptozotocin

Sample fluid	Component:	Percent of total ^{3}H I	II	III	IV	V
Plasma		24.0	2.0	24.2	25.9	23.9
Urine		23.7	7.4	18.2	31.3	19.3
Liver homogenate		> 90	ND†	ND†	(< 10)	

*^{3}H-streptozotocin (300 mg/kg) was injected ip into mice. Plasma and liver samples were obtained 30 min after injection; urine was collected for 4 hrs. The R_f values of components I, II, III, IV, and V were 0.08, 0.28, 0.52, 0.63, and 0.75, respectively.

†ND = not determined.

TABLE 5.—Streptozotocin levels in plasma, urine, and liver of mice treated with ^{3}H-streptozotocin: comparison of assay methods

Tissue	μg Streptozotocin (equivalents)/ml (tissue)*			
	Total ^{3}H	Radiochromatography	Bioassay	Chemical assay
Plasma	182	91	93	89
Urine	6100	3080	2730	3090
Liver	539	50	46	508

*^{3}H-streptozotocin (300 mg/kg) was administered ip to mice. Plasma and liver samples were obtained 30 min after injection; urine was collected over a 4-hr period. The samples were chromatographed and the chromatographic data were used to calculate the actual streptozotocin level in the samples. Streptozotocin levels were also determined by chemical assay and bioassay.

Schein et al (14) determined the level of streptozotocin in plasma and liver by chemical assay. Our results clearly indicate that while there was good correlation between the plasma levels determined by the two assay methods, chemical assay of liver samples gave considerably higher values than the bioassay. Chemical assay also gave higher values than the bioassay when the kidney and pancreas were tested. This indication of extensive metabolism of streptozotocin in the tissues was further confirmed by radiochromatography of tissue samples. Thirty minutes after injection, about 50% of the total radioactivity was still present as streptozotocin in the plasma and urine, while less than 10% was present as streptozotocin in the liver. The streptozotocin metabolites present in the tissue samples have not yet been identified.

Streptozotocin causes diabetes by selective destruction of pancreatic islet beta cells (4). Nicotinamide protects against this action of streptozotocin without reversing the antileukemic action of the drug (13). We also found (unpublished results) that an injection of 200 mg/kg nicotinamide protected against the lethality of a single ip injection of streptozotocin. Nicotinamide, however, did not affect the level of streptozotocin bioequivalents or chemical equivalents present in the kidney or pancreas. Rakieten et al (15) reported that nicotinamide depresses the renal oncogenic action of the drug but at the same time increases the incidence of islet cell tumors. This indicates that the increased incidence of islet cell tumors after treatment with streptozotocin plus nicotinamide could not be due to a greater concentration of the drug in the kidney. These results also show that the total uptake of streptozotocin into the pancreas was the same whether nicotinamide was present or not. However, it is possible that the streptozotocin concentration in the pancreas may not reflect the specific concentration in the islet cells.

Schein has shown that 1-methyl-1-nitrosourea crosses the blood-brain barrier, causes neurologic toxicity, and depresses the nicotinamide-adenine-dinucleotide level in the brain (16). Our results show that ^{3}H-streptozotocin does not enter the brain, confirming a similar observation by Schein (16). Therefore, streptozotocin would not be useful against intracranial and meningeal neoplastic conditions.

Streptozotocin has been found to be useful in the treatment of pancreatic insulinomas (5). Since the bioassay is able to detect about 1 μg/ml of the drug, it could be profitably used to determine streptozotocin levels in human tissues.

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Effect of Levamisole (NSC-177023) on In Vitro Human Lymphocyte Transformation [1,2,3]

David Copeland, Thomas Stewart, and Jules Harris [4,5]

SUMMARY

Levamisole, a drug that is immunostimulatory in both experimental animals and man, neither stimulated resting human lymphocytes in vitro nor augmented or inhibited their responsiveness to autologous cells, specific mitogens, or nonspecific mitogens.

[Cancer Chemother Rep 58:167–170, 1974]

Some immunotherapeutic approaches to the management of cancer have included the use of nonspecific immunostimulatory agents (1). The most popular of these has been bacillus Calmette-Guérin (BCG) vaccine (2,3). Agents with similar or greater activity would be useful for future programs of immunotherapy. Levamisole, a relatively nontoxic compound that has enjoyed some success as an antithelminthic (4), has been found to have immunostimulatory activity when given to animals (5–8). A number of clinical trials have been undertaken to assess its immunopotentiating activity in man. The drug has been shown to enhance the delayed hypersensitivity reactions of patients with cancer (9,10).

In this study, we attempted to determine whether the drug's immunostimulatory activity could be demonstrated in vitro through an action that it might have on resting human lated by either (*a*) specific mitogens (streptolysin-O [SLO], streptokinase-streptodornase [SK-SD], or autologous lymphocyte mixed-lymphocyte reactions [MLR]), or (*b*) nonspecific mitogens (phytohemagglutinin [PHA] or pokeweed mitogen [PWM]). It was thought that the MLR assay would probably provide information regarding the possible activity of the drug in potentiating host response to tumor since the MLR represents, in effect, an in vitro allograft reaction and, as such, it is akin to the response of host lymphocytes to an autochthonous tumor cell with neoantigens (11,12).

MATERIALS AND METHODS

Peripheral venous blood from normal subjects was drawn into plastic syringes moistened with preservative-free heparin (heparin sodium, USP; Fisher, Ottawa, Canada) mixed gently with 2 ml of dextran (6% in saline; Abbott Laboratories, North Chicago, Ill) per 10 ml of blood and incubated for 1 hour at 37 C. The leukocyte-rich plasma was then drawn off. For PHA, SLO, SK-SD, and PWM studies, cultures consisted of 10^6 lymphocytes in 13 × 100-mm round-bottom, screw-top Pyrex glass tubes with 2 ml of Eagle's spinner-modified minimal essential medium (MEM) (Grand Island Biological Co., Grand Island, NY) supplemented with 20 millimols/liter of glutamine and penicillin-streptomycin solution and 1 ml of autologous plasma. Each culture tube con-

[1] Received June 18, 1973; revised Aug 30, 1973; accepted Sept 19, 1973.

[2] Supported by grant 242 from the Ontario Cancer Treatment and Research Foundation.

[3] Levamisole: CAS reg. No. 16595-80-5; imidazo-[2,1-*b*]thiazole, 2,3,5,6-tetrahydro-6-phenyl-, monohydrochloride. Provided by McNeil Laboratories, Toronto, Canada.

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lymphocytes or on human lymphocytes stimutained a volume of 3 ml. For studies of specific and nonspecific mitogen responses, individual 3-ml cultures containing 10^6 lymphocytes were stimulated with either (*a*) 0.05 ml of PHA (phytohemagglutinin-M; Difco Laboratories, Detroit, Mich), (*b*) 0.1 ml of SLO (Difco Laboratories, Detroit, Mich), (*c*) 0.1 ml of SK-SD (Varidase; Lederle Laboratories, Pearl River, NY; preparation reconstituted with 2 ml of saline and used following 24 hours of dialysis), or (*d*) 0.01 ml of reconstituted PWM (Barker and Farnes, Grand Island Biological Co., Grand Island, NY) containing 0.05 mg of PWM.

In one-way MLR studies, responding cells were used in a final concentration of 5×10^5 cells per 3-ml (13 × 100-mm) culture tube. Stimulator cells were treated with 20 μg/ml of mitomycin C[6] (Mann Research, New York, NY) for 30 minutes at 37 C, washed three times, and added to the 3-ml culture tube in a concentration of 5×10^5 cells per tube. A_m and B_m indicate mitomycin C-treated stimulator cells. In two-way MLR studies, non-mitomycin C-treated cells of two donors (A and B) were mixed. Tubes contained 2 ml of medium and 1 ml of plasma from either donor A or donor B (plasma called a and b, respectively).

Levamisole was diluted in MEM so that a final concentration of 10^{-2}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, or 10^{-8} M was obtained in the culture tubes described previously. Control tubes contained no levamisole. The concentration range used was chosen to correspond to serum levels of drug in man obtained with full therapeutic doses of levamisole (9).[7]

Cultures were incubated for 5 days in 5% CO_2 in air at 37 C. Then, after a terminal 3-hour incubation with 2 microcuries of tritiated thymidine (specific activity, 6.7 curies/millimol; New England Nuclear Corporation, Montreal, Canada), the cultures were harvested for liquid scintillation counting as previously described (13).

RESULTS

The results obtained with varying molar concentrations of levamisole in the mixed lymphocyte reaction and with specific and nonspecific mitogenic stimulation of lymphocytes are shown in tables 1 and 2. The figures cited are median values with the range given in parentheses. Cultures from ten normal patients were studied to arrive at these figures. The value for each normal patient represents the mean of triplicate determinations. Variation about the mean is ± 10%. At a molar concentration of 10^{-2}, levamisole suppresses in vitro lymphocyte reactivity. Most of the cells maintained in culture for 5 days at this concentration are dead. At concentrations ranging upward from 10^{-4} to 10^{-8} M, the drug neither stimulates resting lymphocytes nor augments or inhibits responsiveness to autologous cells, specific mitogens, or nonspecific mitogens.

The methods described in this paper are techniques to study the in vitro functional integrity of the human lymphocyte. They do not lend themselves to an evaluation of the in vivo mechanism of action of levamisole.

[6] Mitomycin C: NSC-26980; CAS reg. No. 50-07-7.

[7] Brugmans J. Personal communication.

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TABLE 1.—Effect of levamisole on mixed lymphocyte reactivity

Cells†	Liquid scintillation count* after treatment with levamisole at a molar concentration of—					
	Control	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-8}
Two-way MLR studies:						
A + B in *a* plasma	49,261 (3174–70,566)	0	49,252 (4503–53,390)	53,344 (5264–68,764)	55,498 (2861–62,108)	54,726 (7323–74,385)
A + B in *b* plasma	54,057 (4942–79,625)	0	63,686 (5883–69,511)	55,040 (7196–68,557)	53,796 (5501–76,298)	48,870 (2370–48,870)
One-way MLR studies:						
A_m + B in *b* plasma	23,095 (8160–31,768)	0	11,640 (4203–40,081)	17,331 (12,591–31,924)	20,818 (3579–37,755)	25,050 (12,974–43,198)
A + B_m in *a* plasma	29,679 (5922–56,272)	0	21,699 (4590–29,751)	29,909 (6956–44,345)	29,464 (5768–32,765)	19,125 (7968–27,722)

*Figures are median values; range is given in parentheses.
†A_m and B_m indicate mitomycin C-treated stimulator cells. In two-way MLR studies, non-mitomycin C-treated cells of two donors (A and B) were mixed. *See* text for further explanation.

TABLE 2.—Effect of levamisole on the responsiveness of human lymphocytes to specific and nonspecific mitogens

Mitogen†	Liquid scintillation count* after treatment with levamisole at a molar concentration of—						
	Control	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
SLO	32,155 (13,127–102,383)	0	24,826 (4967–99,448)	25,939 (8278–97,046)	34,028 (10,263–65,567)	22,313 (16,084–66,769)	36,003 (18,675–69,993)
SK-SD	27,965 (18,018–107,177)	0	24,442 (10,907–84,901)	24,929 (3377–85,652)	29,460 (17,725–87,798)	28,266 (0–87,519)	30,490 (18,074–38,379)
PHA	37,338 (19,532–138,353)	0	33,557 (21,401–115,230)	37,949 (13,942–100,693)	39,847 (18,825–111,709)	32,034 (29,636–111,824)	37,242 (19,732–69,700)
PWM	72,531 (27,791–152,924)	0	79,126 (25,088–166,051)	75,555 (34,565–148,048)	73,130 (28,398–159,960)	90,121 (74,713–147,941)	62,569 (36,572–103,695)
Spontaneous (unstimulated)	325 (150–6620)	152 (0–263)	397 (299–3303)	—	403 (130–4813)	—	367 (261–2940)

*Figures are median values; range is given in parentheses.
†SLO = streptolysin-O; SK-SD = streptokinase-streptodornase (Varidase); PHA = phytohemagglutinin-M; PWM = pokeweed mitogen.

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Prevention of Hemolysis Induced by Ellipticine (NSC-71795) in Rhesus Monkeys [1,2,3]

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SUMMARY

The intravenous administration of ellipticine (1.5–12.0 mg/kg), an antitumor alkaloid, was found to produce hypotension, bradycardia, and an increase in carotid artery blood flow in rhesus monkeys. Doses of 3.0–12.0 mg/kg, when dissolved in 1N HCl and adjusted to pH 4.0 or 6.0, produced varying degrees of hemolysis. Ellipticine exerted immediate hemolytic activity with maximal red blood cell lysis within 5 minutes. After 15 minutes the amount of detectable hemolyzed blood declined. The amount of hemolysis increased substantially between doses of 6.0 (79 ± 7 mg/100 ml hemochromogen) and 12.0 mg (320 ± 7 mg/100 ml hemochromogen), indicating that a critical drug concentration exists between these two doses. In the present experiments the hemolysis induced by the 12-mg/kg dose was prevented when ellipticine was prepared in citrate buffer, 300 milliosmoles (pH 4.0), or 2.5% sodium edetate (pH 4.0). The mechanism of hemolytic action did not appear to be due to the binding of calcium since hemolysis was also absent when ellipticine was prepared in a 2.5% calcium disodium edetate solution. Even though hemolysis was prevented, ellipticine still produced hypotension, bradycardia, and an increase in carotid artery blood flow.

[Cancer Chemother Rep 58:171–179, 1974]

Ellipticine, an alkaloid isolated from the Australian plant *Excavatia coccinea*, exerts antitumor activity against certain experimental neoplasms (1). The structure of the compound is shown in figure 1. Preliminary toxicity studies have revealed that intravenous (iv) administration of ellipticine produces alterations in blood pressure, heart rate, and the electrocardiogram (ECG) (2). Hemolysis was also present immediately after the injection of ellipticine (3). The cardiovascular effects could be partially ameliorated by pretreatment with various pharmacologic agents (4). A similar study to prevent ellipticine-induced hemolysis has not been attempted. The present studies, in rhesus monkeys, were initi-

[1] Received July 5, 1973; revised Aug 30, 1973; accepted Sept 19, 1973.

[2] Supported in part by contract NO1-CM-33728 from the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3] Ellipticine: CAS reg. No. 519-23-3; 6*H*-pyrido[4,3-*b*]carbazole, 5,11-dimethyl-. Supplied by Drug Research and Development, DCT, NCI.

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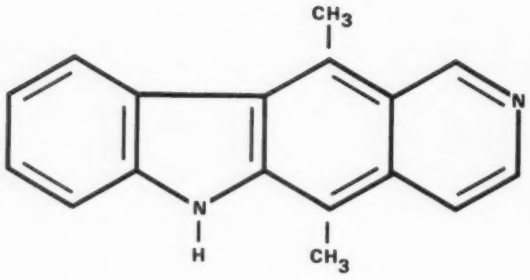

ELLIPTICINE

FIGURE 1.—Structure of ellipticine.

ated to examine the extent of hemolysis induced by ellipticine and, if possible, to devise a means of reducing this undesirable action which would limit the clinical usefulness of this compound.

MATERIALS AND METHODS

In all of the experiments, male rhesus monkeys (3.5–4.5 kg) were anesthetized with pentobarbital sodium (30 mg/kg) and secured to an animal operating table. Polyethylene catheters were inserted into the left femoral vein, left femoral artery, right femoral artery, and trachea.

Hemolytic Activity of Ellipticine at Various Doses

Ellipticine was administered iv at doses of 1.5, 3.0, 6.0, and 12.0 mg/kg over a 3.5–4.5-minute period. The compound was initially dissolved in 1N HCl and the pH was adjusted to 6.0 with 2N NaOH. The final volume for the various doses ranged from 8 to 10 ml with an osmolarity between 302 and 388 milliosmoles. Each dose was given to three monkeys. In two other monkeys 10 ml of the solute (1N HCl adjusted to pH 6.0 with 2N NaOH) was infused over a 4-minute period. Prior to and at 1, 5, 15, 30, 60, 90, and 120 minutes after injection, blood samples (4 ml) were collected from the right femoral artery. Glass tubes, without anticoagulant, were allowed to fill spontaneously. Catheters were kept free of clots by periodic flushing with 1–2 ml of normal physiologic saline. Two hours after the injection all samples were centrifuged (780 *g*) and the amount of hemolysis was determined by a benzidine method (5). A 0.1-ml portion of each serum sample was mixed with 2 ml of benzidine reagent (2 g benzidine HCl in a solution of 60 ml of distilled water, 100 ml of 95% ethyl alcohol, and 40 ml of glacial acetic acid) and 1 ml of 0.6% hydrogen peroxide in a glass test tube. In the instances where considerable hemolysis had occurred the initial sample of 0.1 ml of serum was diluted with 0.9 ml of physiologic saline, and 0.1 ml of this diluted serum was then mixed with benzidine reagent and hydrogen peroxide. After 60 minutes, 20 ml of 20% glacial acetic acid was added to each tube and the intensity of the color measured in a Beckman DB spectrophotometer at a wavelength of 515 nm.

Cardiovascular Activity of Ellipticine at Various Doses

Arterial blood pressure, from the left femoral artery, and respiration were obtained from appropriate pressure transducers connected to a Hewlett-Packard polygraph. Blood flow was measured by means of a 2.0-mm Biotronex flow probe inserted around the right carotid artery and connected to a Biotronex electromagnetic flowmeter. The lead II ECG was obtained by means of needle-tipped electrodes which were inserted into the limbs; heart rate was determined by means of a cardiotach preamplifier. These parameters were continuously recorded during the 2-hour blood-sampling period.

Hemolytic Activity of Ellipticine Prepared in Various Solutes

Ellipticine (12.0 mg/kg) was initially dissolved in 4 ml of 1N HCl and, prior to administration, was prepared in a variety of solutions.

Citrate buffer.—The constituents of the buffer, sodium citrate (14.5 g) and citric acid (20.8 g), were each dissolved in 500 ml of distilled water. Citrate buffer (300 milliosmoles, pH 4.0) was obtained by mixing 1.7 ml of the sodium citrate solution with every 3.3 ml of the citric acid solution. The ellipticine (12 mg/kg)/HCl (4 ml) solution was added to 5 ml of the citrate buffer. The pH was adjusted to 4.0 giving a final dose volume of ap-

proximately 10 ml. The osmolarity of the ellipticine/citrate buffer solution was approximately 300 milliosmoles.

Sodium edetate[7] *(tetrasodium ethylenediaminetetraacetate; sodium EDTA).*—Sodium edetate was prepared as a 50-mg/ml solution in distilled water. Sodium edetate (7.5 ml) was added to 6 ml of ellipticine (12 mg/kg)/HCl solution. The pH of this solution ranged between 5.5 and 6.0, and in some instances the solution turned slightly cloudy. Even after the pH was lowered to 4.0 with 1N HCl and the volume was adjusted to 15 ml with normal physiologic saline, a slight cloudiness persisted. The final sodium edetate concentration of the solution was 2.5%. The solutions were clarified by centrifugation (780 *g*) and 10 ml (dose volume) of the supernate was utilized for injection.

Calcium disodium edetate[8] *(calcium disodium versenate; calcium disodium EDTA).*—This EDTA was available as a commercial preparation (200 mg/ml) and 1.88 ml (375 mg) was added to 12 ml of ellipticine (12.0 mg/kg)/HCl solution. A slight cloudiness appeared and persisted even after the pH was reduced to 4.0 with 1N HCl. The final volume was adjusted to 15 ml with normal physiologic saline (2.5% calcium disodium edetate, final concentration). The solution was clarified by centrifugation and 10 ml of the supernate was utilized for injection. The osmolarity of both ellipticine/EDTA solutions was approximately 600 milliosmoles.

Polyvinylpyrrolidone[9] *(PVP).*—Ellipticine (6.25 mg/ml) in a 15% PVP solution was administered iv at a dose of 6.0 mg/kg in a volume of approximately 4 ml.

The various ellipticine solutions in doses of 12.0 or 6.0 mg/kg were infused iv over a 4-minute period. The administration of each preparation of ellipticine was repeated in five separate experiments. One additional animal was injected with ellipticine dissolved in HCl (adjusted to pH 4.0 with 2N NaOH), whereas another animal was given 10 ml of the HCl solute adjusted to pH 4.0 with 2N NaOH. In all experiments blood was collected and the amount of hemolysis determined in a manner similar to that previously described in this paper.

Cardiovascular Activity of Various Ellipticine Preparations

The physiologic parameters mentioned previously were monitored in all experiments before and up to 2 hours after ellipticine administration. At the end of the experimental period all animals were killed by an overdose of pentobarbital sodium.

RESULTS

Hemolytic Activity of Ellipticine at Various Doses

Ellipticine caused a dose-dependent destruction of red blood cells within 1 minute after completion of the injection (fig 2). Peak effects were noted to occur within 1–5 minutes after completion of the injection, after which the amount of detectable hemochromogen decreased at each time interval. A dose of 12.0 mg/kg produced peak levels of 320 ± 7 mg/100 ml hemochromogen, whereas 79 mg/100 ml was the maximum occurring after the 6.0-mg/kg dose. Relatively slight increases (14 mg/100 ml) were detected after the 3.0-mg/kg dose and no significant change was seen after the lowest dose (1.5 mg/kg). No increase in hemochromogen concentration occurred when the HCl solute (adjusted to pH 6.0) was given.

Cardiovascular Activity of Ellipticine at Various Doses

Ellipticine initially caused changes in certain cardiovascular parameters. The effects of the various doses (1.5–12.0 mg/kg) on mean arterial pressure, heart rate, and carotid artery blood flow are summarized in figure 3. A dose-dependent hypotensive response occurred within 1 minute after injections of 3.0 (–14%) to 12.0 mg/kg (–59%) of ellipticine. In all instances the pressure returned to or near control levels by 5 minutes and no further significant

[7] Supplied by K + K Laboratories, Plainview, NY.
[8] Supplied by Riker Laboratories, Inc, Northridge, Calif.
[9] Supplied by University of Michigan Pharmacy, Ann Arbor.

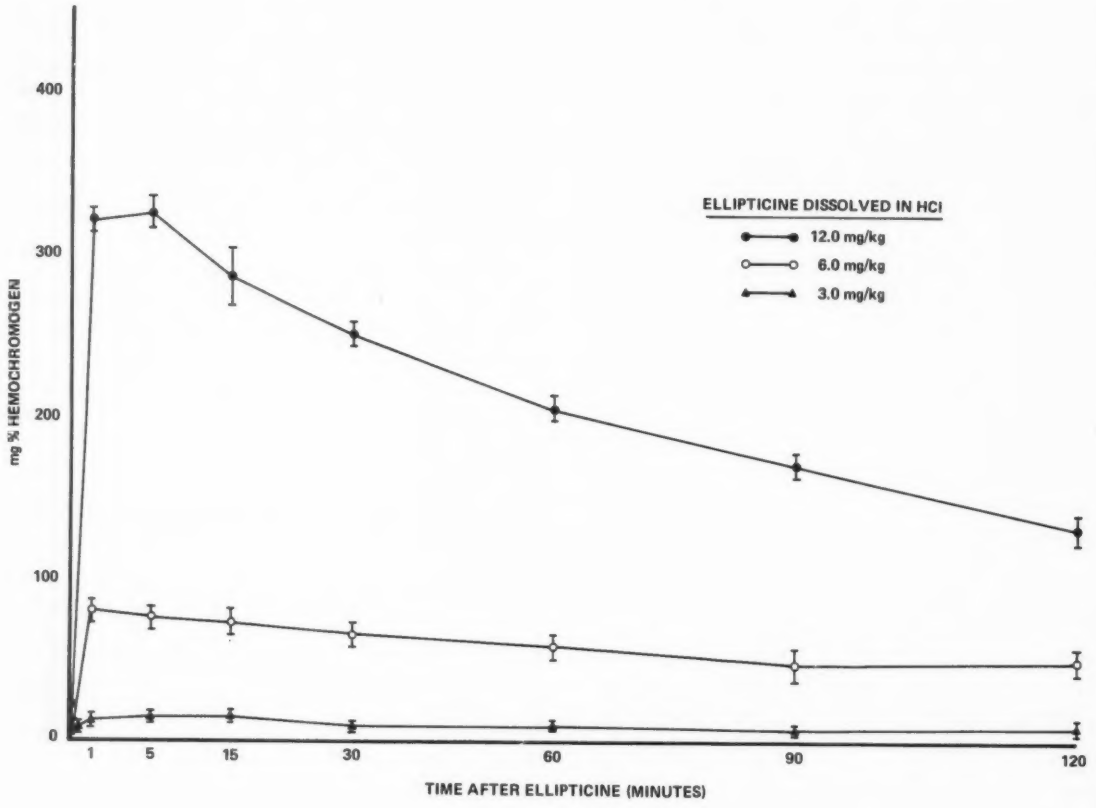

FIGURE 2.—The amount of hemolysis, determined as mg/100 ml hemochromogen, detected in serum from the anesthetized rhesus monkey at various times after 4-min iv infusions of 3.0, 6.0, or 12.0 mg/kg of ellipticine dissolved in 1N HCl and adjusted to pH 6.0 with 2N NaOH. Each point represents the mean ± SE from three separate experiments.

changes occurred during the remainder of the experiments.

A similar initial dose-dependent bradycardia ensued with the 3.0-mg/kg dose, depressing the heart rate on an average of nine beats per minute, whereas decreases of 25–32 beats per minute occurred after the doses of 6.0 and 12.0 mg/kg. Although some recovery was noted, the rate remained below control levels for over 60 minutes after the two higher doses.

All doses of ellipticine caused immediate increases (+31%–63%) in carotid artery blood flow. These increases were of short duration, since within 5–15 minutes flow returned to control levels in all of the animals except those receiving the lowest dose (1.5 mg/kg). In these animals flow remained slightly elevated for the 60-minute duration of the experiments.

The various doses of ellipticine exerted only minor ECG and respiratory effects. In some experiments the amplitude of the R-wave was slightly diminished for ½–3 minutes. A slight increase in depth of respiration was noted, but this effect did not persist beyond the injection period.

Hemolytic Activity of Ellipticine in Various Formulations

A dose of ellipticine of 12.0 mg/kg dissolved in HCl and adjusted to pH 6.0 with 2N NaOH produced an average of 320 ± 7-mg/100 ml hemochromogen level by 1 minute after injec-

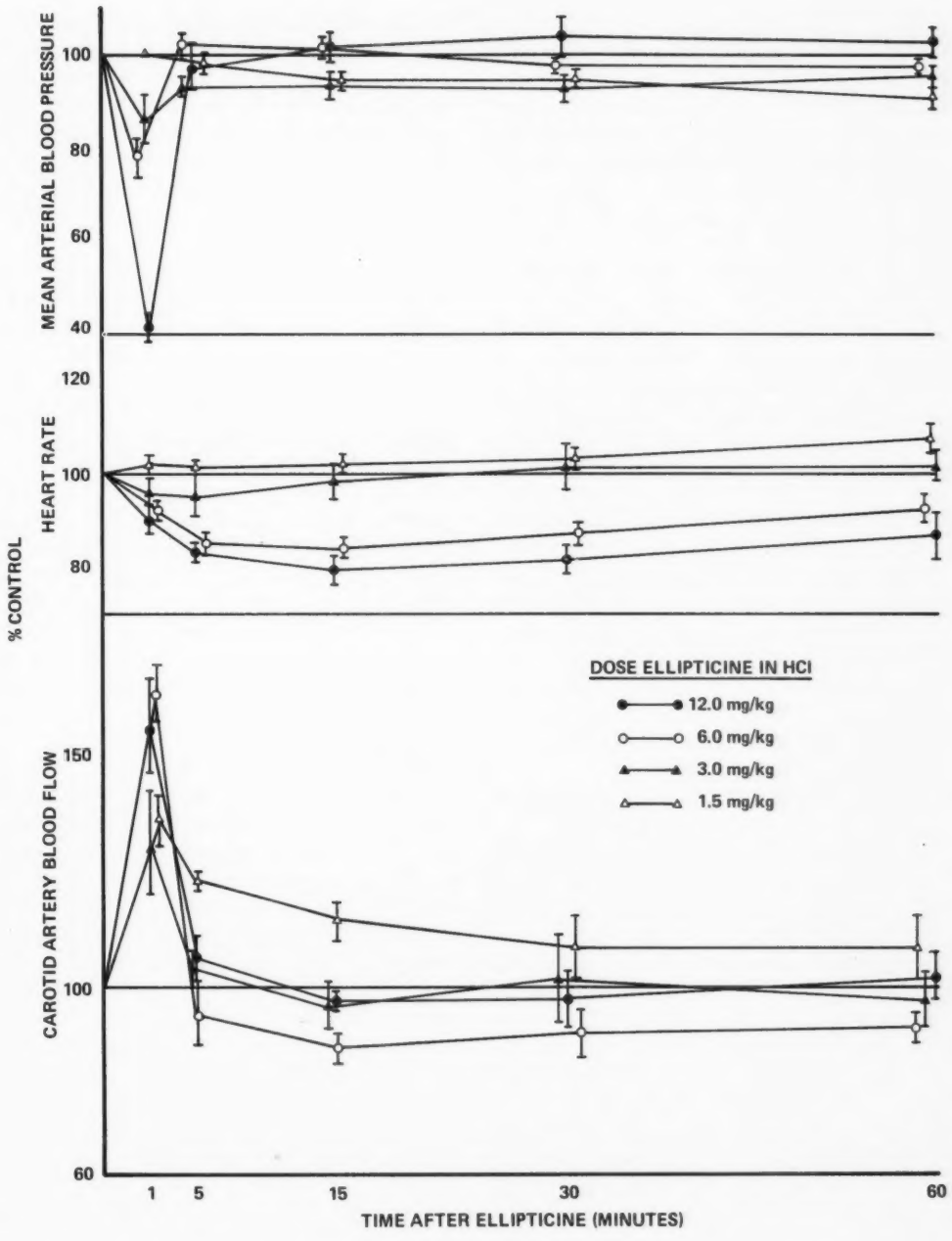

FIGURE 3.—The effect of 4-min iv infusions of 1.5, 3.0, 6.0, or 12.0 mg/kg of ellipticine (dissolved in 1N HCl and adjusted to pH 6.0 with 2N NaOH) on mean arterial pressure, heart rate, and carotid artery blood flow in the anesthetized rhesus monkey. Values obtained were converted to percent of control and each point represents the mean ± SE from three separate experiments.

tion in three separate experiments. In another experiment a similar dose of ellipticine, dissolved in HCl but adjusted to pH 4.0 with 2N NaOH, induced a 316-mg/100 ml hemochromogen level. The time course for this peak response and the gradual decline in hemochromogen concentration were identical to those of the HCl/ellipticine solution at pH 6.0. The pH 4.0 HCl solute (adjusted with 2N NaOH) caused no increase in hemochromogen in one additional experiment.

When doses of 12.0 mg/kg were prepared to pH 4.0 in citrate buffer, sodium edetate, or calcium disodium edetate, only minimal amounts of hemochromogen were detected at any time interval (fig 4). The average hemochromogen concentration was not significantly changed from the pre-injection level when the dose of 12.0 mg/kg of ellipticine was dissolved in either citrate buffer or 2.5% sodium edetate. A slight increase in hemochromogen from 5.2 to 19.3 mg/100 ml occurred when ellipticine was in the calcium disodium edetate solution.

The preparation of ellipticine in PVP offered no advantage over ellipticine dissolved in HCl since both formulations at 6.0 mg/kg produced equivalent amounts of hemolysis over a similar time course (fig 4, top).

Cardiovascular Activity of Ellipticine in Various Formulations

A dose of 12.0 mg/kg of ellipticine (dissolved in HCl) caused an immediate decrease in blood pressure (–40 mm Hg), a decrease in heart rate (–14 beats/min), and an increase in carotid artery blood flow (+50%). Similar responses occurred when ellipticine was prepared in citrate buffer, sodium edetate, and calcium disodium edetate (fig 5). Blood pressure recovered more completely in those animals given the ellipticine/HCl solution. The initial hypotension and bradycardia were less severe in animals given the ellipticine/calcium disodium edetate. In these animals blood pressure completely returned to normal within 5 minutes but then gradually declined over the next 60 minutes.

All formulations increased carotid artery blood flow. The smallest increase occurred with the calcium disodium edetate (+27% ± 2%) and the largest with the citrate buffer solution (+98% ± 18%). A rapid decline in this parameter occurred in all experiments within 5 minutes, and by 15 minutes flow was at or slightly below control levels in animals given the HCl or calcium disodium edetate solutions of ellipticine. The flow stabilized slightly above the control level with the sodium edetate solution whereas a secondary increase began after 30 minutes in animals given the ellipticine/citrate buffer solution.

The HCl-formulated ellipticine caused a minor decrease in the amplitude of the R-wave and produced a slight increase in depth of respiration. This latter effect was also noted with the other ellipticine formulations, however the ECG effects were in some instances more profound. In six of nine experiments, in which the citrate buffer/ellipticine, calcium disodium edetate/ellipticine, or sodium edetate/ellipticine solutions were administered, a change in the ECG pattern ensued due to marked decreases in R-wave and increases in S-wave amplitude. Some alterations in amplitude of the T-wave were also noted and in one case a ventricular arrhythmia appeared. In all instances a normal ECG pattern reappeared within 7–24 minutes.

DISCUSSION

Ellipticine was found to produce varying degrees of hemolysis at doses of 3.0–12.0 mg/kg. The amount of hemolysis increased substantially between 6.0 and 12.0 mg/kg, indicating that a critical concentration exists in that dose interval. In other experiments, human red blood cells exposed to ellipticine in vitro also were hemolyzed (6). In these preliminary studies surface tension measurements demonstrated that the critical micellar concentration of ellipticine corresponds to the drug concentration at which extensive hemolysis occurred. These authors concluded that the extent of hemolysis correlated well with the surface activity, lipophilic property, and cellular uptake of ellipticine. The most extensive hemolysis in the rhesus monkey occurred at a dose of 12 mg/kg. Assuming a 6%–7% blood volume in the 4-kg monkey, this dose nearly corresponds (0.81×10^{-3}M) with the 10^{-3}M of the in vitro experiments. A rapid decline in the hemolytic activity in vitro was noted at 10^{-4}M; likewise, considerably less hemolysis occurred

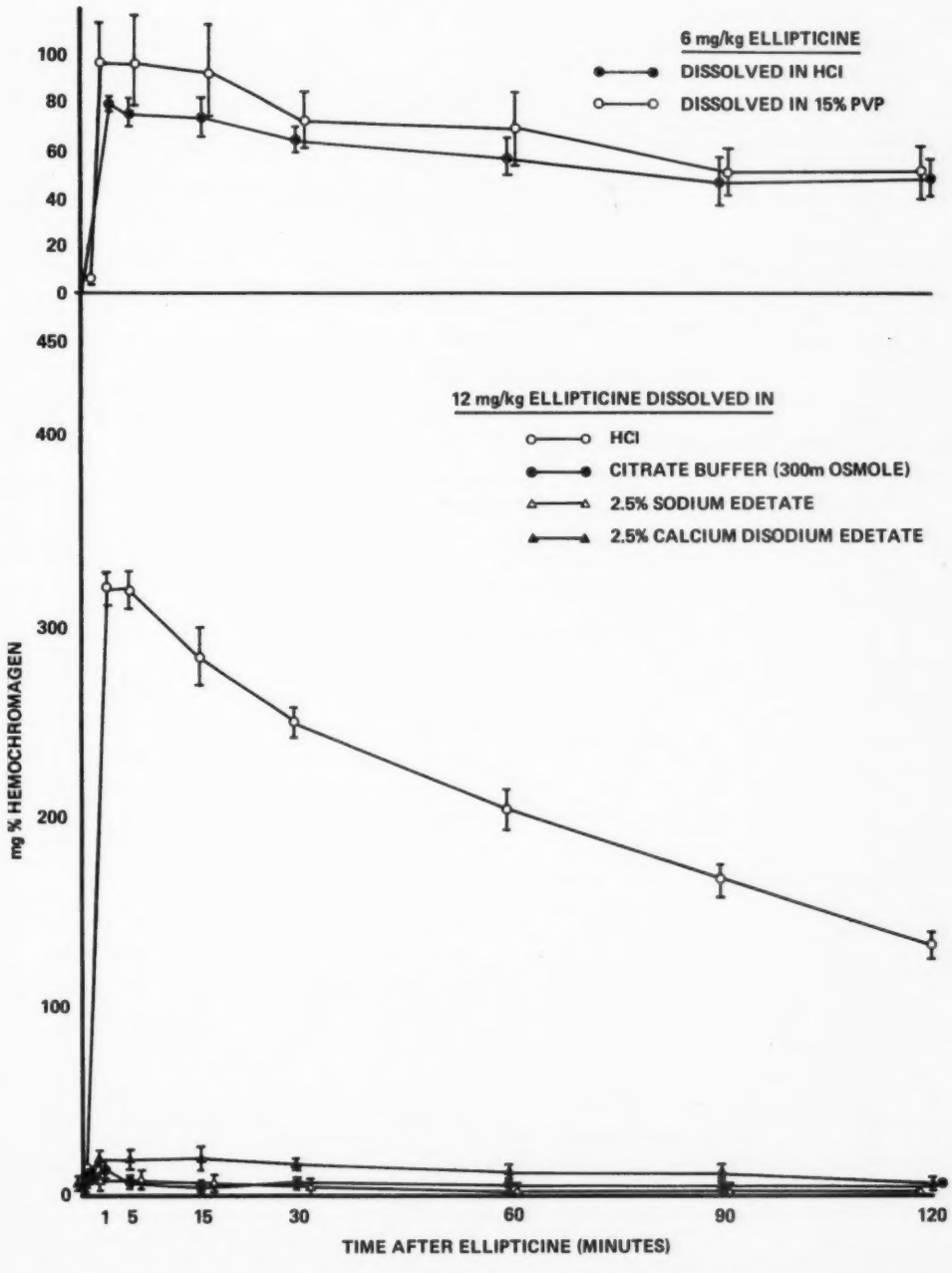

FIGURE 4.—Comparison of the amount of hemolysis, determined as percent hemochromogen, detected in serum from the anesthetized rhesus monkey at various times after 4-min iv infusions of (*a*) 6.0 mg/kg of ellipticine (dissolved in 1N HCl and adjusted to pH 6.0 with 2N NaOH) or 6.0 mg/kg of 15% PVP (top portion); (*b*) 12.0 mg/kg of ellipticine dissolved in 1N HCl (adjusted to pH 6.0 with 2N NaOH), citrate buffer (300 milliosmoles; pH 4.0), 2.5% sodium edetate (adjusted to pH 4.0 with 1N HCl), or 2.5% calcium disodium edetate (adjusted to pH 4.0 with 1N HCl). Each point represents the mean ± SE from three separate experiments.

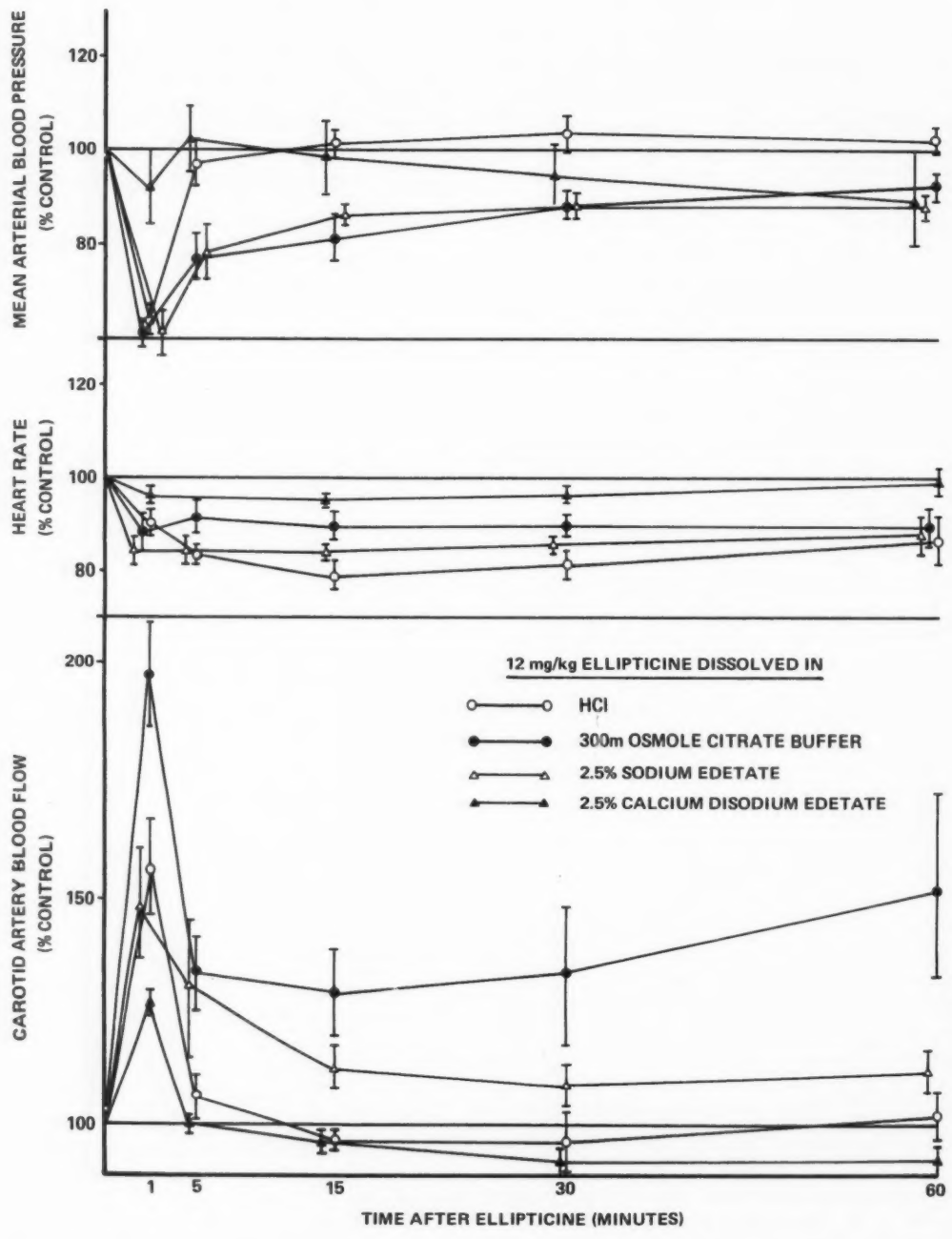

FIGURE 5.—Comparison of the effects of 4-min iv infusions of 12.0 mg/kg of ellipticine dissolved in either 1N HCl (adjusted to pH 6.0 with 2N NaOH), citrate buffer (300 milliosmoles; pH 4.0), 2.5% sodium edetate (adjusted to pH 4.0 with 1N HCl), or 2.5% calcium disodium edetate (adjusted to pH 4.0 with 1N HCl), on mean arterial pressure, heart rate, and carotid artery blood flow in the anesthetized rhesus monkey. Values obtained were converted to percent of control and each point represents the mean ± SE from three separate experiments.

in vivo as the dose was reduced to less than 6.0 mg/kg.

In the present experiments the marked hemolysis induced by the dose of 12 mg/kg was almost entirely prevented when ellipticine was prepared with citrate buffer or sodium edetate. These two agents share a common property in that both are capable of forming poorly dissociable chelate complexes with calcium ions. Calcium ions are firmly bound on the red blood cell membrane and recent evidence suggests that calcium-lipid interaction in the cell membrane may be extremely important in regulating cell flexibility and permeability (7). The prevention of ellipticine-induced hemolysis does not appear to be due to the binding of calcium since this activity also occurred when calcium disodium edetate was used, and this agent does not lower the concentration of ionic calcium in the plasma (8). It is possible that the binding of trace elements may be involved in the protection against ellipticine hemolysis (9). In vitro experiments have demonstrated that citrate buffer and sodium edetate inhibit the uptake of ellipticine by the red blood cell (6). Thus, it appears that the citrate buffer and the two EDTA preparations may alter membrane permeability to ellipticine directly or by forming a complex with ellipticine and thereby retarding uptake. EDTA is ionized and would, therefore, not be expected to penetrate into the cell. At the present time it is not known whether the cellular uptake of ellipticine is retarded in other types of cells, other than the red blood cell. It should be noted that the formulation of ellipticine in citrate does not alter the antitumor activity of the compound in experimental animal systems (Davignon JP, personal communication).

Ellipticine has been found to produce various cardiovascular actions. The 12.0-mg/kg dose (HCl), which elicited the most hemolysis, caused hypotension, bradycardia, and an increase in carotid artery blood flow. These cardiovascular responses were altered only quantitatively when this dose of ellipticine was prepared in the other three formulations. Less hypotension occurred with the ellipticine/calcium disodium edetate, and the increase in carotid artery blood flow was greater after the ellipticine/citrate buffer solution. The various preparations of ellipticine tended to produce more drastic ECG alterations than did the HCl solution; thus, the cardiovascular actions of ellipticine were still present in spite of the inhibition of hemolysis.

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In Vitro Studies on Macromomycin (NSC-170105): Effects on Cultured TA3Ha and L1210 Cells [1,2]

Muriel M. Lippman [3]

SUMMARY

TA3Ha and L1210 cells are prevented from further division for 24–72 hours by a single addition of 1–2 μg/ml of macromomycin (MCR) to the culture medium. At this cytostatic concentration the cells remained viable and reproductive capacity could be restored by removal of the MCR by brief treatment of the cells with trypsin. Cells did not recover their ability to divide by simple resuspension in fresh medium. A large percentage of the treated population had twice the cell volume and many giant cells were found. Evidence is presented which indicates that DNA synthesis is not blocked at cytostatic concentrations of MCR.

[Cancer Chemother Rep 58:181–187, 1974]

Chimura et al (1) reported that injection of macromomycin (MCR), a 15,000 molecular weight protein obtained from filtrates of cultures of *Streptomyces macromyceticus*, prolonged the life of mice bearing L1210 leukemia or Sarcoma 180. Studies in our laboratory confirm the activity of MCR on L1210 ascites leukemia (2) and show it to be active against P388 leukemia, B16 melanoma, and Lewis lung carcinoma as well (report submitted for publication). The activity of MCR, shown by Chimura et al (1) and by Kunimoto et al (3) to be due to binding to the cell surface, is supported by studies which demonstrated increased immunogenicity of MCR-treated cells (2).

Kunimoto et al (3) studied the effect of MCR on HeLa cells and Yoshida sarcoma cells in culture. They found DNA synthesis to be partially blocked by addition of concentrations of MCR which were lethal to synchronized HeLa cells in less than 2 hours. The studies reported here concern the activity of MCR on cultured TA3Ha and L1210 cells and establish the nature of effects on these cells in lower concentrations which are cytostatic rather than cytotoxic.[4] It is shown by cytochemical study that MCR produces profound alterations in the organization of DNA but that at cytostatic concentrations of MCR synthesis of DNA is not prevented.

MATERIALS AND METHODS

The TA3Ha cultured cell line was derived from a 7-day-old TA3Ha ascites tumor carried

[1] Received Aug 14, 1973; revised Dec 6, 1973; accepted Dec 20, 1973.

[2] Macromomycin: CAS reg. No. 12634-34-3. Submitted for testing to Drug Research and Development (DR&D), Division of Cancer Treatment (DCT), National Cancer Institute (NCI), by Bristol Laboratories, Syracuse, NY.

[3] Drug Evaluation Branch, DR&D, DCT, NCI, Bethesda, Md.

[4] The use of the terms "cytostatic" and "cytotoxic" is subject to varied interpretation. The publication by Federoff (on the proposed usage of animal culture terms) suggests that the term "cytotoxic" be defined by the particular investigator (4). Accordingly, in this paper "cytostatic" is used to designate agents which produce loss of cell division activity without killing the cells. "Cytotoxic" is used to designate an agent which causes cell death. Failure of the cells to exclude trypan blue is the criterion used for death. Therefore, an agent which prevents cells from dividing for one or more cell cycles but does not kill the cells during that period of division arrest is cytostatic not cytotoxic.

in A/He mice. The tumor originated as a spontaneous mammary adenocarcinoma in 1949 in the laboratory of Dr. T. Hauschka. Detailed characterization and history of the tumor are given elsewhere (5). The culture is maintained in spinner flasks in our laboratory in RPMI 1630 medium (formulation listed in Grand Island Biological Co. catalog) supplemented with 10% fetal calf serum (Colorado Serum Co.) and penicillin-streptomycin. Cells used for experiments were in 10–55 culture passages, during which time the population retained a constant doubling time of nearly 24 hours.

The L1210 leukemia cells are long established in culture and were maintained in spinner flasks under the same culture conditions as the TA3Ha cells. The population doubling time for these cells is approximately 16 hours under these conditions.

Cell counts were made with the Model B Coulter counter and size distribution was plotted on the Model H plotter.

Trypsin solution supplied by the Grand Island Biological Co. (0.25%) was used throughout the trypsinization experiments. Cells were spun down and resuspended in spinner flasks in fresh, serum-free medium containing trypsin at a concentration of .025%. The cells were trypsinized for 5–10 minutes at 37 C and serum was added to stop trypsin activity. The trypsin-containing medium was removed and the cells were resuspended in fresh medium containing serum. Cell viability was tested before and after trypsinization by trypan blue exclusion which was used routinely to test viability.

MCR was prepared immediately before initial use in Hanks' solution or RPMI 1630 medium, refrigerated, and used for up to 1 week for experiments.

Feulgen staining for DNA was carried out as described by Merchant et al (6).

RESULTS

Cytostatic Action on TA3Ha Cells

The effect of adding MCR to asynchronous, exponentially growing cultures of TA3Ha cells is shown in figures 1 and 2. Figure 1 shows that in this experiment a concentration of 2 μg/ml was effective in completely preventing division, while 1 μg/ml was only partially effective. The results of three separate experiments using 1 μg/ml are shown in figure 2. In these experiments 1 μg/ml was effective in blocking division. The variability in response (1–2 μg/ml) may be due to the time MCR was kept in solution prior to use in culture. MCR tends to be somewhat unstable according to H. Umezawa (personal communication) and Bristol Laboratories (unpublished data). All cells continued to exclude trypan blue for the entire period of the assay (48 or 72 hours), but since the cell count was reduced slightly in the treated populations the question of viability was still open and was, therefore, tested in another way.

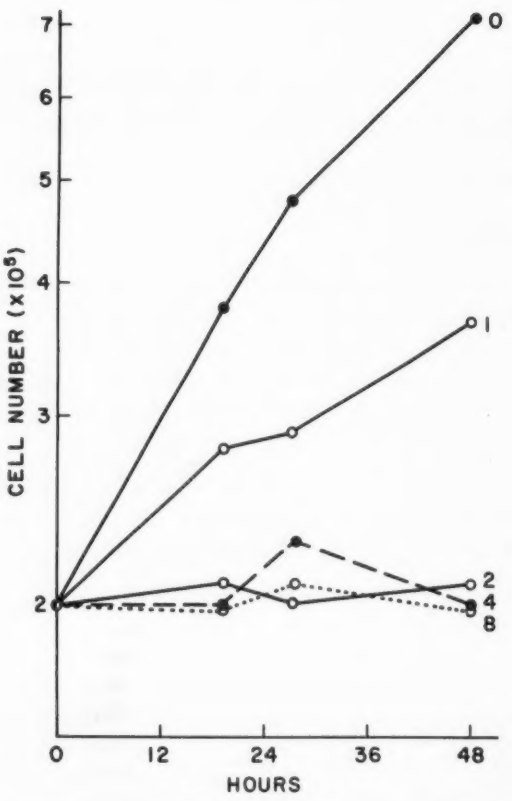


FIGURE 1.—Addition of 1, 2, 4, or 8 μg/ml of MCR to spinner cultures of TA3Ha cells at time 0.

After 72 hours in the presence of 1 μg/ml of MCR the culture was trypsinized as described under Materials and Methods. The trypsinized

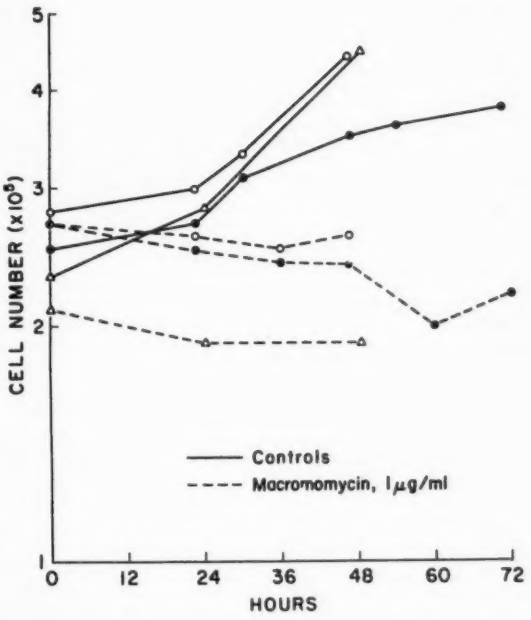


FIGURE 2.—Three separate experiments. Addition of 1 μg/ml of MCR to spinner cultures of TA3Ha cells at time 0.

cells were resuspended in fresh medium at a concentration of 0.55 × 10^5 cells/ml. Control cells were treated the same way but were suspended at somewhat higher concentrations (these cells were used for another study requiring a higher concentration). Viability at resuspension was over 95% for both treated and control cultures. Figure 3 shows that the reproductive capacity of the population was restored and the population doubled at the normal rate for at least 24 hours. Since the cells still retained their reproductive capacity after the 72 hours of their division arrest by 1 μg/ml of MCR, this concentration of MCR was cytostatic but not cytotoxic. Other proof of viability involved tumor formation with appropriate median survival times following injection of these cells into A/He mice (2). It can be concluded that 1–2 μg/ml of this MCR preparation is cytostatic to TA3Ha cells in culture but that it is not cytotoxic at this concentration.

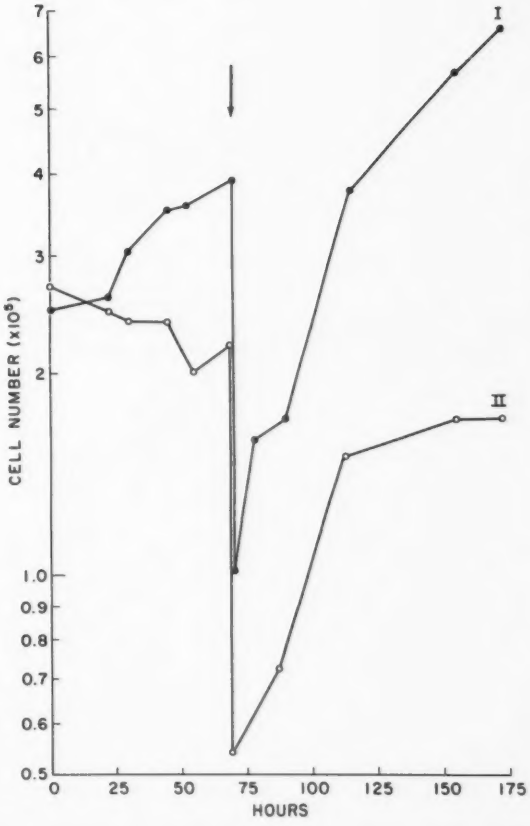


FIGURE 3.—MCR (1 μg/ml) added to spinner cultures of TA3Ha cells. At 72 hours both control and MCR-treated cultures were trypsinized at 37 C for 5 min and resuspended in fresh medium for further growth. I=control culture; II=MCR-treated culture.

Cytostatic Action on L1210 Cells

Similar studies were carried out with cultured L1210 cells. Figure 4 shows that L1210 cells are prevented from dividing for 48 hours by 1 μg/ml of MCR.

Two experiments were carried out in order to determine whether simple washing and resuspending of the cells in MCR-free medium after 48 (fig 4) or 96 (fig 5) hours failed to restore reproductive activity. In figure 5 it should be noted that resuspension of the cells in fresh medium failed to restore even the slight degree of reproductive activity seen before resuspension.

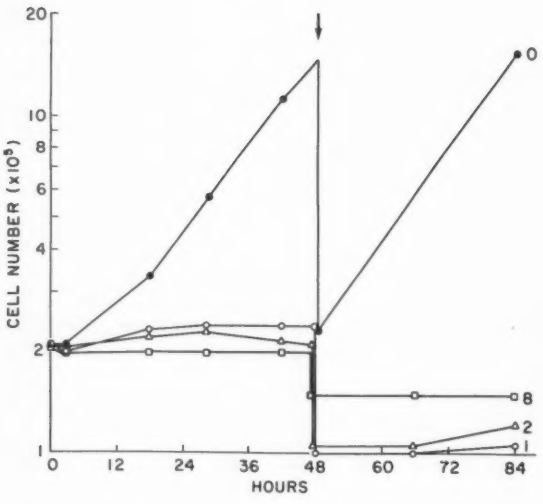


FIGURE 4.—Addition of 1, 2, or 8 μg/ml of MCR to spinner cultures of L1210 cells. At 48 hours cells were centrifuged and resuspended in fresh medium.

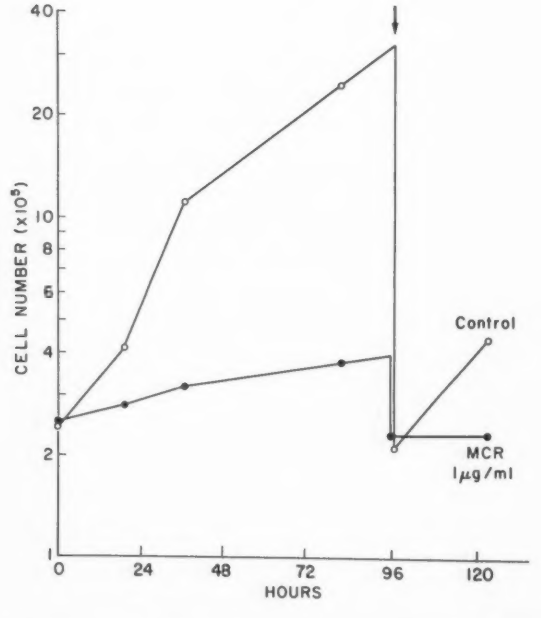


FIGURE 5.—Addition of 1 μg/ml of MCR to spinner cultures of L1210 cells. At 96 hours cells were centrifuged and resuspended in fresh medium.

The fact that L1210 cells do not die for at least 24 hours as a result of exposure to 1 μg/ml of MCR is shown by a trypsinization experiment. Cells exposed to the agent for 2 or 24 hours were trypsinized and then put into fresh medium. Table 1 shows that after trypsinization the cells recovered their reproductive capacity for as long as 48 hours in the case of a 2-hour exposure to the drug and for about 24 hours after a 24-hour exposure to the drug. In one instance the viability was maintained for 48 hours and in the other for 72 hours. It is not clear whether ultimate loss of viability is due to unusual vulnerability of the MCR-treated L1210 cells to the effects of trypsin or a result of cellular changes due to the MCR itself. The ability of the cells to recover any reproductive capacity after division arrest proves that the cells were not killed during the period when they were exposed to the MCR.

Cytologic Changes in Cells Treated With MCR

TA3Ha cells treated with MCR are greatly enlarged. The size distribution of control MCR-treated cells is shown in figure 6. While the average increase in diameter of the cells was twice that of control cells, a large part of the population consisted of giant cells as much as 40 times the volume of control cells. Some of these giant and enlarged cells are shown in figures 7 and 8.

Feulgen staining revealed numerous abnormalities in the organization of DNA in these enlarged cells. Among these were frequent appearance of abortive mitotic figures (fig 7 [*d*, *f*, and *h*]), fragmented and irregular condensations of DNA (fig 7 [*e* and *f*], fig 8 [*f*]), diffuse Feulgen-positive material in the cytoplasm (fig 7 [*f* and *h*], fig 8 [*b*, *c*, *d*, and *f*]), and numerous irregularities in nuclear structure (fig 7 [*c*, *e*, and *g*], fig 8 [*b*, *c*, *d*, *e*, and *f*]). In addition to these abnormalities there was uniformly more DNA in each of the treated cells. None of these abnormalities appear in control cells simultaneously stained (fig 7 [*a* and *b*], fig 8 [*a*]). Quantitative data concerning the DNA content of these cells based on cytofluorographic and microdensitometric analysis will be reported elsewhere.

The increase in DNA content accompanied by blockade of cell division and no apparent

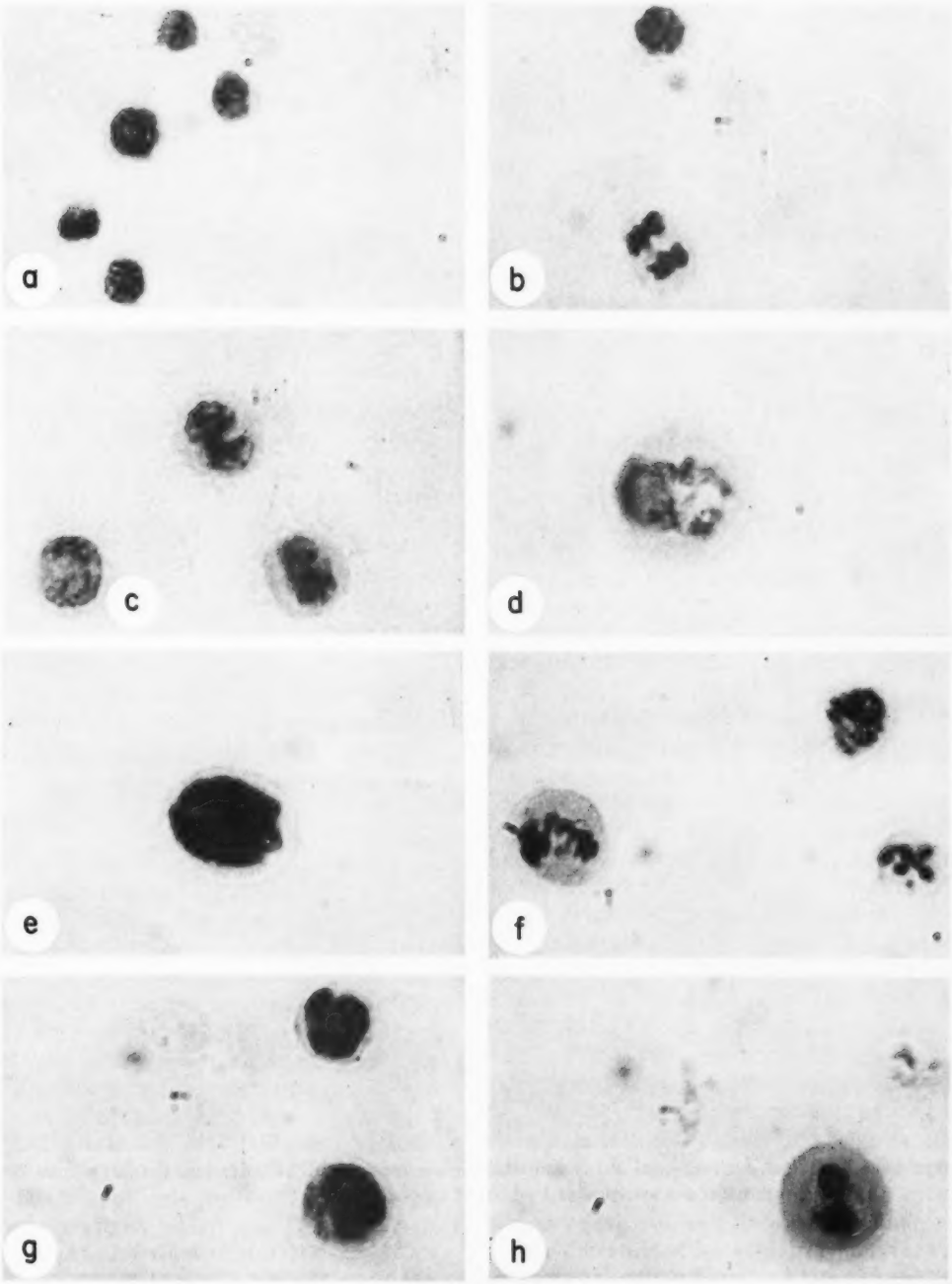


FIGURE 7.—Feulgen-stained control (*a*, *b*) and macromomycin-treated cells (*c–h*). Smears prepared 24 hours after the addition of 1 μg/ml of macromomycin (× 320).

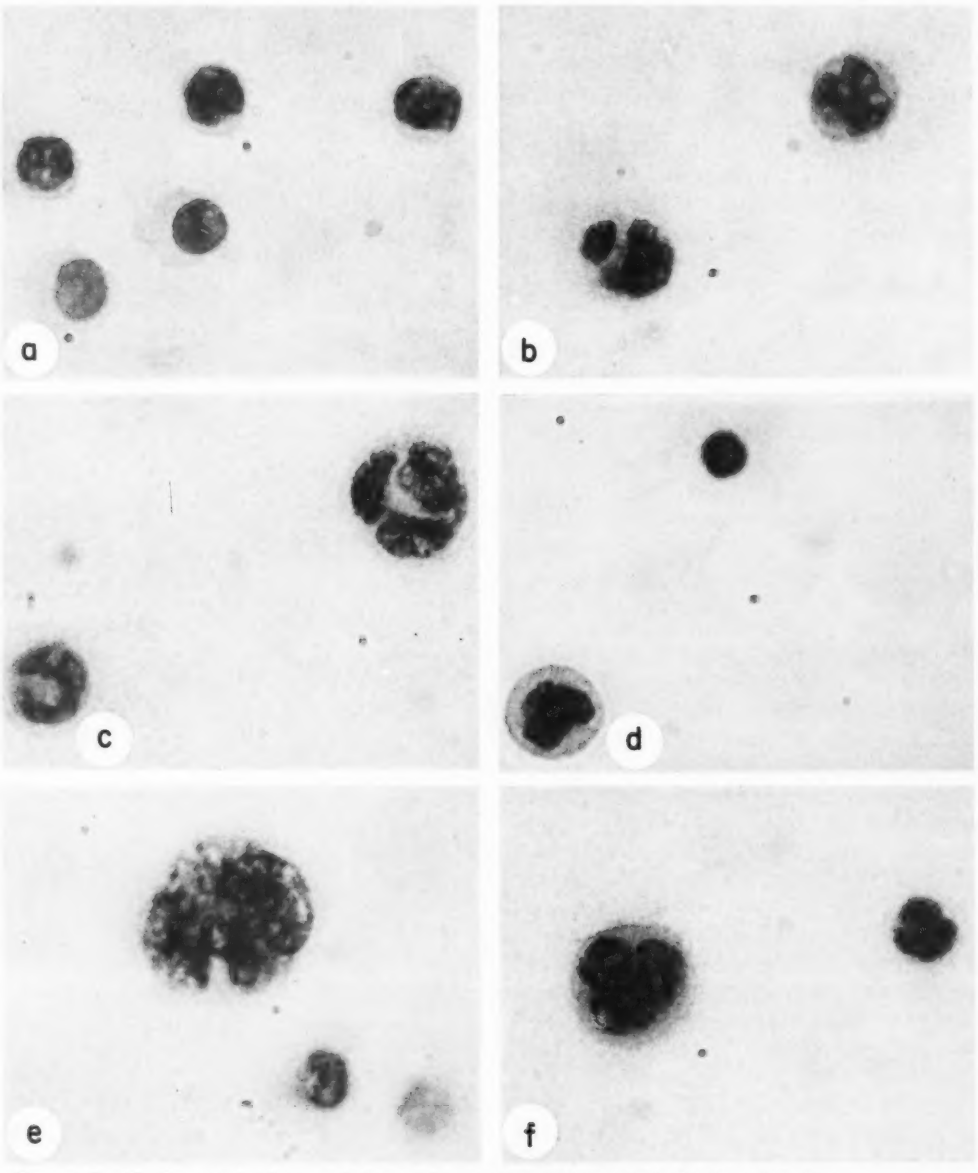


FIGURE 8.—Feulgen-stained control (*a*) and macromomycin-treated (*b–f*) cells. Smear prepared 24 hours after the addition of 1 μg/ml of macromomycin (× 320).

TABLE 1.—Release of cytostatic block of L1210 cells exposed to 1 μg/ml of MCR for 2 or 24 hours

Duration of MCR exposure at 1 μg/ml	Cell count (×10³) after trypsin release*			
	10 min	24 hrs	48 hrs	72 hrs
2 hrs	2.0	4.7	6.3	7.1†
24 hrs	2.4	7.1	‡	‡
Control: no MCR	2.0	6.2	20.0	20.0

*Viability >95% unless otherwise indicated.
†Viability 5%.
‡Viability 30%.

increase in mitotic figures suggest a G_2 block. At the same time the very intense staining and massive amounts of DNA observed in the enlarged and giant cells suggest that cells had continued periods of DNA synthesis without cleavage and consequently are likely to be polyploid. Based on these observations there does not appear to be blockage to DNA synthesis when cytostatic concentrations are used; on the contrary, DNA synthesis seems to be prominently operative.

All of the features observed with the TA3Ha cells, including enlargement, high content, and abnormal organization of the DNA, were also characteristic of MCR-treated L1210 cells.

DISCUSSION

The present report concerning the direct action of MCR on tumor cells shows that 1–2 μg/ml is cytostatic but not cytotoxic to 2×10^5 cells/ml of TA3Ha or L1210 cells in vitro for periods as long as 72 hours. The cytostatic action produces enlarged and giant cells with unusually high content of DNA suggesting that the MCR blocks the cells in G_2 or early mitosis but that DNA synthesis remains unblocked leading to endoreduplication.

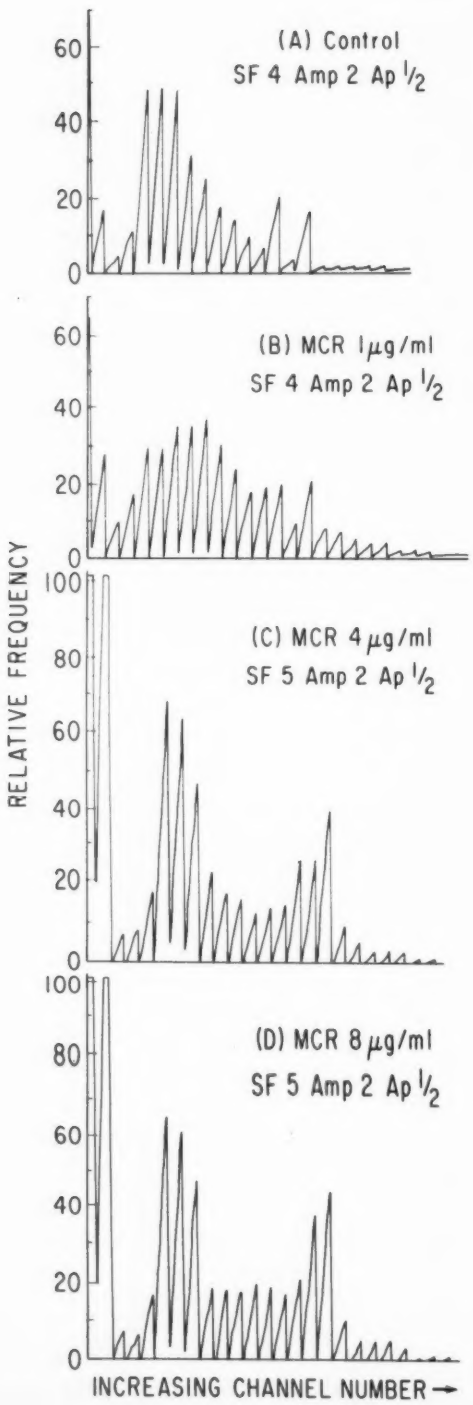
High concentrations of MCR are cytotoxic. Kunimoto et al (3) demonstrated 50% reduction in DNA synthesis by 90 minutes with MCR concentrations which are lethal to cells by 2 hours. At the time that DNA synthesis was reduced no effect was observed on RNA and protein synthesis. This sequence of events is not surprising since cells can remain viable for varying and extended periods of time in the absence of DNA synthesis but not in the absence of protein synthesis. The converse is not necessarily true, namely, that DNA synthesis proceeds normally in dying cells. The question is then whether the observed reduction in DNA synthesis was a reflection of incipient cell death rather than a direct cause. This is an important distinction to be made in evaluating the nature of the effects of any particular agent, because rational choices on combinations of agents depend upon accurate information as to their mechanisms of action.

A similar situation occurs with other agents, such as demecolcine[5] and related alkaloids. It is well known that these agents prevent cells from dividing by interfering with assembly of microtubules which form the mitotic apparatus. The result of this is failure of cells to pass beyond metaphase with consequent accumulation of cells in metaphase. In such cases endoreduplication leading to polyploidy is not uncommon (7). It has also been reported that DNA synthesis is reduced by demecolcine (8–10). This is inconsistent with the observed polyploidy and is most likely attributable to the doses used or the duration of the experimental observation.

It is not, therefore, uncommon to find conflicting reports concerning the primary biochemical or cytologic changes produced by a given agent. It is apparent that some standardization in testing should be used. Description and measurement of biochemical and cytologic lesions relative to cell division blockade would best be determined for cells in which the reproductive capacity is impaired but trauma to the point of death has not occurred.

In the case of in vitro experiments with MCR three different actions of the agent occur and may lead to cell death: (*a*) direct application

[5] Demecolcine: NSC-3096; CAS reg. No. 477-30-5; colchicine, *N*-deacetyl-*N*-methyl-; Colcemid.


(A) Control
SF 4 Amp 2 Ap 1/2
(B) MCR 1 μg/ml
SF 4 Amp 2 Ap 1/2
(C) MCR 4 μg/ml
SF 5 Amp 2 Ap 1/2
(D) MCR 8 μg/ml
SF 5 Amp 2 Ap 1/2
RELATIVE FREQUENCY
INCREASING CHANNEL NUMBER →

of high concentrations are cytotoxic, (*b*) cells may be arrested in G_2 or later stages of the division cycle and ultimately disintegrate without further division, (*c*) cells may undergo endoreduplication leading to the formation of polyploids which may or may not retain the capacity to divide. The trypsin experiments showed that when MCR is removed further division may occur, but among the aneuploid population such division may be frequently abortive. Instances of successful division of such cells may lead to permanent aneuploid populations. Such populations, usually polyploids, have been frequently produced by colchicine (7)[6] and are known to occur spontaneously (11).

Other agents are known to block cell division in G_2 or to block division while permitting endoreduplication (12,13). MCR appears to be unique in that these effects are exerted by virtue of the drug's adsorption to the cell surface (3) and can be reversed by removal from the surface.

MCR has demonstrated antitumor activity. Two separate actions of the agent may be involved in the in vivo activity: increased immunogenicity of the cells exposed to MCR (2) and direct cytostatic or cytotoxic action on the tumor cells. Both actions are related to the ability of the MCR to adsorb to the surface of the tumor cells. Studies are presently in progress to determine whether MCR exhibits any selectivity in its binding to the cell surface.

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[6] Colchicine: NSC-757; CAS reg. No. 7006-15-7.

FIGURE 6.—Coulter counter plots of size distribution of control and MCR-treated cells 21 hours after addition of the indicated dose of MCR. Increasing channel number indicates increasing size. SF=scale factor and sets the relative frequency scale so that the total number of cells counted gives a readable height on the chart. Amp (amplitude) and Ap (aperture) settings are set for particular cell types so that the size range fits within the threshold setting limits. Using the same Amp and Ap settings means that the position of the channel in each of the four measurements is numerically comparable.

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Drug-Tumor Sensitivity Matching Procedure Using Diffusion Chambers In Vivo [1,2]

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SUMMARY

C3H/HeJ mice bearing intraperitoneally (ip) implanted millipore diffusion chambers (0.45μ pore size) containing L1210 leukemia cells were treated with a variety of antitumor agents. As evidence of drug effectiveness against the cultured leukemic cells it was required that an agent produce cytopathogenic effects or reduce tumor population by greater than 90% in relation to untreated control cultures. The drugs proving effective in such tests were those that significantly extended life in conventional ip implanted L1210 screening tests. The drug sensitivities of in vivo cultures of cells from C3H/HeJ spontaneous mammary tumors were determined in the same way. First-generation transplants of the spontaneous tumor were used to demonstrate that agents selected by the diffusion chamber drug-tumor sensitivity matching procedure could affect the tumor growth rate. Positive responses in the diffusion chamber tests provide presumptive evidence for the presence of the biochemical mechanisms necessary for drug sensitivity in tumors. It is suggested that intrinsically, drug-sensitive tumor populations might provide false negative responses because of cytokinetic, pharmacologic, or toxicologic factors. Agents active against diffusion chamber-cultured C3H/HeJ mammary tumor cells were given in a cyclic regimen to mice with first-generation transplants of this tumor. Two courses of cyclic treatment (cyclophosphamide, hydroxyurea, vincristine, and 6-mercaptopurine) administered to mice with moderate (0.3 cm^3) tumors halted tumor volume increase for the treatment period; with the same treatment larger tumors (1.2 cm^3) continued to grow. When cyclic treatment was started 24 hours after tumor implantation, tumors failed to appear in 31%–37% of the treated animals within twice the mean survival time of the untreated controls.

[Cancer Chemother Rep 58:189–205, 1974]

The logical application of cancer chemotherapeutic agents in the clinic requires that drug, tumor, and host be matched at the biochemical, cytokinetic, pharmacologic, and toxicologic levels (1–4). Unfortunately, there

[1] Received Sept 28, 1973; accepted Oct 25, 1973.

[2] Supported in part by the Medical Research Council of New Zealand, the Auckland Division of the Cancer Society of New Zealand, Inc., and a grant from the Auckland Hospital Board.

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[5] We are grateful to Drs. J. McLean and W. McIndoe, National Women's Hospital, for invaluable assistance in obtaining the clinical tumor specimens.

is seldom adequate knowledge available at any of these levels when treatment of an individual's cancer is initiated. It is possible to envisage certain practical methods of circumventing this lack of knowledge. For example, a demonstration of the intrinsic drug sensitivity of an individual's cancer cells could substitute for matching via a detailed knowledge of intracellular biochemical pathways and those necessary for drug action. Attempts to accomplish successful prematching of tumor and agents have been many and varied but no single method has been sufficiently successful to have been generally adopted. The bulk of these attempts suffer from the non-physiologic conditions used and the application of drug levels at times which bear little relationship to the drug levels and dosing times observed in patients. Often, activity against tumor cells in these tests has been gauged by indirect biochemical methods; seldom has there been a direct assessment of the desired tumor cell death. One notable exception, less subject to the above criticisms, is the approach described by Heckmann (5) in which human tumor cells were maintained in diffusion chambers implanted intraperitoneally (ip) in rats. Evidence of cytopathogenic effects on tumor cells after drug treatment of the animal was taken as predicting positive drug responses. To us, the major attractions of such a system were that (*a*) drug effects on the target cell species were directly observed, and (*b*) the essential triumvirate of drug, tumor, and host were maintained. The buffer of the substitute host in these experiments ensures that drug levels and dosing times lie within realistic limits. Activation, degradation, and excretion of a drug by the substitute host continually modify drug blood levels by processes comparable to those of a patient. The seeming advantages of drug-tumor matching utilizing diffusion chamber-cultured specimens have led us to investigate a variant of this method. In the diffusion chamber culture experiments reported in this paper mice were used as tumor hosts,[6] and the majority of the available antitumor agents were selected with, or demonstrated to act against, experimental tumors in mice.

Preliminary experiments with clinical samples showed that the conditions inside millipore diffusion chambers (5–7) implanted ip in mice were compatible with the extended survival of human cancer cells. Some of the conventional antitumor agents listed in table 1 could produce cytopathogenic changes in the human cells included in these chambers. However, it was realized that attempts to obtain evidence of a correlation between the ability of an agent to produce cytopathogenic changes in chamber-cultured cells and the ability of that drug to produce significant clinical response would, of necessity, involve a long-term program. Further, many factors at the clinical level, notably the dosages and treatment schedules employed, would not be under our control, and attempted correlations could fail because of inadequate control of these essential factors. Accordingly, we have examined the experimental animal tumors available to us to determine if the ability of drugs to induce cytopathogenic changes in chamber-cultured tumor cells corresponds with the ability of those drugs to inhibit tumors growing progressively in their normal hosts.

METHODS AND MATERIALS

Diffusion chambers were constructed from nontoxic Plexiglass rings (Millipore Catalog No. Pr 00 014) to which two gridded membranes (Millipore HAWG 013 00) were fixed with the supplier's specified adhesive. The membranes were attached so that one had its gridded surface on the outside of the chamber and the opposing membrane had its ruled surface on the inside. Chambers were washed and sterilized in one operation. The washing step is valuable for removing traces of detergent used in membrane manufacture. The plastic rings are heat distortable and cannot be satisfactorily autoclaved. Assembled chambers can be sterilized with ethylene oxide but a further washing step is still desirable. By

[6] The substitute host plays a dominant role in attempted drug-tumor matching using diffusion chambers. If rats were used as hosts the high blood levels of deoxycytidine could make it impossible to determine the sensitivity of chamber-cultured tumor cells to cytosine arabinoside (8–10). Since mice have played a major role in selecting current cancer chemotherapeutic agents, they should receive first consideration for the role of substitute host.

soaking in an aqueous solution of penicillin sodium G (50 units/ml), streptomycin (100 μg/ml), kanamycin (100 μg/ml), and amphotericin B (25 μg/ml) for 4 hours, washing and sterilizing steps were conveniently combined in one operation. Any liquid remaining in the washed chambers was aspirated with a hypodermic needle. The washed chambers were loaded into petri dishes and dried and stored in vacuo in a desiccator over silica gel.

Many different diffusion chamber designs have been described (11–13),[7] and certain of these appear to have attractive features. Because we started our studies using already available millipore chambers, it was decided to complete preliminary experiments with these despite the considerable cost of the components. We would prefer to use the more robust nuclepore membranes in place of the millipore membranes but have not found a suitable mountant which will allow ready examination by transmitted light.

Tumor Cell Suspensions

L1210 and L1210/63878[8] leukemia cell suspensions were obtained in the usual way from ip inoculated DBA_2/J carrier mice.

Spontaneous mammary tumors shelled from elderly C3H/HeJ female breeders were sliced into approximately 4-mm slices and added to freshly thawed 199 medium (3–5 C).[9,10] The tumor slices were reduced between two scalpel blades to cubes of approximately 3 mm on each side, the tumor fragments were suspended in trypsin diluent[9] (trypsin concentrate [12.5%][9] added to a concentration of 0.25%), and the flask was swirled at 37 C for 20 minutes. After a rapid coarse filtration (100-mesh nylon) the enzymatic digest was cooled to 0–5 C. Remaining solid portions of tumor were returned to the digestion flask and treated further with trypsin as described above, successive digestions being continued until no further useful number of cells were dislodged.

Tumor cells were collected by centrifugation (100 *g* for 10 min) and the cells were suspended in 199 medium[9,10] to which 10% fetal calf serum[9] had been added. Approximately 1.5 ml of medium /0.1 ml of packed cells was used. On the basis of hemocytometer counts (with the aid of trypan blue) suitable dilutions were made so that there was a final concentration of 5×10^6 viable tumor cells/ml.

Chamber Loading

Chambers were filled with tumor cell suspensions (0.15 ml) and sealed in the usual way (11–13).[11] The filled diffusion chambers were placed in an ice-cooled petri dish containing a 4-mm deep layer of 199 medium containing 10% fetal calf serum, ensuring that the membrane with the gridded surface visible lay uppermost.

Mice (DBA_2/J, C3H/HeJ [Jackson Laboratories, 1971]) weighing 18.5–22.5 g were anesthetized with chloral hydrate (400 mg/kg) injected ip in aqueous solution. DBA_2 male mice proved very susceptible to chloral hydrate and effective anesthetic doses invariably killed some of the animals. In this latter case sodium pentobarbitone (29 mg/kg) produced acceptable anesthesia but of somewhat shorter duration.

Chambers were implanted ip (two per mouse). If care was taken that the incisions through the skin and peritoneum did not overlap, suturing proved unnecessary. Each filled diffusion chamber was removed from where it had been held in cold medium (half-turned, so that the membrane with its ungridded face visible was uppermost) and was inserted into the peritoneum of an upturned mouse so that the chamber lay on the midline against the stomach wall. A second chamber was implanted alongside in the same fashion, ensuring that both lay on the midline of the animal. The animals were then turned and laid face downwards and a prophylactic dose of streptomycin-penicillin mixture (500 μg and 500 units respectively

[7] Collected abstracts compiled by Millipore Corp., Boston, Mass.

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[8] L1210/63878 is an L1210 variant resistant to cytosine arabinoside. We are extremely grateful to Mr. I. Wodinsky, Arthur D. Little, Inc., Boston, Mass, for his courtesy in providing this variant.

[9] Supplied by Commonwealth Serum Laboratories, Parkville, Victoria, Australia.

[10] All media were supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml).

[11] *See* footnote 7.

TABLE 1.—Results of diffusion chamber experiments

Agent	Dose (mg/kg/dose)	Schedule	L1210 leukemia chambers* Cell counts (± SE) × 10^{-3}: C†/T	C–T/C (%)‡	ILS§	L1210/63878 chambers* Cell counts (± SE) × 10^{-3}: C†/T	C–T/T (%)‡	ILS§	C3H mammary chambers* Cell counts (± SE) × 10^{-3}: C†/T	C–T/T (%)‡	C3H¶
Actinomycin D	0.5	Once	8.8±2.2 / 0.8±0.4	91	32	7.9±2.8 / 0.6±0.3	92	36	9.4±3.1 / 0.6±0.2	92	+
Bleomycin	40	Once	8.6±2.6 / 7.6±3.1	12	—**	8.3±2.7 / 7.0±3.2	16	—	9.5±2.9 / 7.8±3.2	18	
Busulfan	10	Once	8.6±2.6 / 6.9±2.4	20	—	7.9±2.8 / 6.9±2.9	13	—	9.7±3.2 / 7.9±3.1	19	
Chlorambucil	5	Once	7.8±2.7 / 0.7±0.4	91	34	7.9±2.8 / 0.6±0.2	92	39	9.4±3.1 / 7.6±3.3	19	
Cyclophosphamide	150	Once	8.8±2.2 / 0.1±0.06	99	92(2)	8.1±3.1 / 0.08±0.03	99	102(1)	9.4±3.1 / 0.02±0.01	99.9	+
Cytosine arabinoside	22	q3h × 8	8.5±1.9 / 0.25±0.1	97	79	8.2±2.1 / 7.2±3.2	12	—	9.7±3.2 / 8.7±2.6	10	−
Dexamethasone	40	Once	8.8±2.2 / 7.2±3.4	14	—	8.3±2.7 / 7.9±3.1	15	—	9.5±2.9 / 10.2±4.1	−7	
Daunorubicin	4.6	Once	8.6±2.6 / 0.8±0.3	91	31	7.9±2.8 / 0.7±0.3	91	39	9.5±2.9 / 0.8±0.3	92	+
Diethylstilbestrol	10	Once	7.8±2.7 / 7.7±3.0	11	—	8.2±2.1 / 7.9±3.1	4	—	9.7±3.2 / 8.6±3.7	11	
5-Fluorouracil	250	Once	8.9±3.1 / 0.7±0.3	92	47	7.7±2.3 / 0.7±0.3	91	52	9.4±3.1 / 7.9±3.9	16	
Fluoxymesterone	50	Once	7.8±2.7 / 8.3±3.3	−6	—	8.2±2.1 / 8.0±3.6	3	—	9.7±3.2 / 8.9±3.2	8	
Hydroxyurea	100	q3h × 8	8.5±1.9 / 0.3±0.14	96	82	8.2±2.1 / 0.3±0.1	96	87	9.7±3.2 / 0.2±0.09	98	+
Mechlorethamine	15	Once	7.8±2.7 / 0.7±0.3	91	42	8.1±3.1 / 0.6±0.2	93	36	10.1±3.8 / 8.7±3.6	14	
Medroxy-progesterone	100	Once	7.8±2.7 / 7.4±3.2	5	—	8.2±2.1 / 7.6±3.1	7	—	9.7±3.2 / 8.5±3.1	12	
Malengestrol acetate	200	Once	7.8±2.7 / 6.9±3.3	12	—	8.1±3.1 / 6.7±2.9	17	—	9.5±2.9 / 7.8±3.4	18	

Melphalan	2.5	Once	8.9±3.1 / 0.6±0.24	93	71	7.9±2.8 / 0.5±0.3	94	84	9.4±3.1 / 7.2±3.4	21	
6-Mercaptopurine	250	Once	8.5±1.9 / 0.7±0.26	92	48	8.2±2.1 / 0.6±0.3	93	42	8.9±2.7 / 0.8±0.3	91	+
Methotrexate	125	Once	8.5±1.9 / 0.5±0.3	94	106	7.7±2.3 / 0.4±0.23	95	97	9.9±2.4 / 7.4±2.9	26	
Mithramycin	800 μg	Once	7.9±2.6 / 5.8±2.8	26	—	7.7±2.3 / 5.3±2.4	31	—	9.4±3.1 / 7.8±3.1	17	
Mitomycin C	3	Once	8.8±3.1 / 0.7±0.3	92	37	8.3±2.7 / 0.7±0.3	92	31	9.4±3.1 / 7.5±3.0	20	
Nitromin	25	Once	8.9±3.1 / 0.6±0.27	93	32	8.1±3.1 / 0.7±0.3	91	37	10.1±3.8 / 9.0±3.1	11	
NSC-29215	0.3	Once	8.9±3.1 / 0.2±0.1	98	64	7.9±2.8 / 0.3±0.17	96	57	9.4±3.1 / 7.3±3.5	22	
NSC-122819	15	Once	8.6±2.6 / 0.08±0.03	98	138(1)	7.7±2.3 / 0.06±0.03	99	122(2)	8.9±2.7 / 7.5±3.0	16	
NSC-150440††	21	Once	8.8±2.2 / 0.1±0.04	99	71	8.1±3.1 / 0.07±0.03	99	68	9.4±3.1 / 0.2±0.07	98	+
Prednisone	500	Once	8.8±2.2 / 0.8±0.5	90	31	8.3±2.7 / 0.7±0.3	92	27	9.5±2.9 / 7.5±3.2	21	
Procarbazine	250	Once	6.9±2.1 / 0.6±0.2	91	49	7.7±2.3 / 0.6±0.3	92	37	9.9±2.4 / 6.8±2.9	31	
Progesterone	500	Once	8.8±2.2 / 8.9±3.4	−12	—	8.1±3.1 / 6.9±2.9	15	—	9.7±3.2 / 8.1±3.6	17	−
Vinblastine	1.5	Once	7.4±2.9 / 0.7±0.3	91	35	8.2±2.1 / 0.8±0.3	90	31	9.9±2.4 / 0.7±0.3	93	+
Vincristine	1.9	Once	7.4±2.9 / 0.6±0.3	92	29	8.2±2.1 / 0.6±0.3	93	33	9.9±2.4 / 0.6±0.3	94	+

*Diffusion chamber membrane cell counts quoted are mean figures per high power field (× 400).

†C = cell count of untreated control membranes; T = cell count of drug-treated membranes.

‡Percentage tumor cell "kill" produced by drug treatment.

§Percent increase in lifespan obtained at the dose and for the schedule quoted against L1210: 10^5 cells were implanted ip in $(C3H/DBA_2)F_1$ hybrids and drug was given 24 hours after tumor implantation. Number of animals surviving 50 days after drug treatment is given in parentheses.

¶Agents can produce statistically significant inhibition of tumor growth rate of the C3H mammary carcinoma.

**− = ILS <25%.

††NSC-150440 is an experimental agent that originated in this laboratory.

in 0.1 ml) was administered intramuscularly into the right thigh. Implanted in this fashion the two chambers lay behind one another in the midline of the animal with the visible gridded membrane surface of the chamber uppermost. The chamber settling time in the medium, and then later in the animals recovering from the anesthetic, is sufficient to ensure (in all the cases we have examined) that greater than 90% of the tumor cells settle and adhere firmly to the lower membrane face. It is then a simple matter to assess the contents of the chambers since only the lower membrane (ungridded face visible) need be removed and examined.

Cytology Studies

Chambers recovered from animals that had been killed were first sponged gently with a saline-saturated tissue to remove adhering mouse cells. They were then fixed in 10% formol saline for at least 30 minutes. The lower (ungridded surface visible) membrane was removed from the fixed chamber with a scalpel blade. The detached membrane was clipped to a microscope slide and processed with standard Papanicolaou staining sequence.

Extensive growth trials were carried out with both L1210 leukemic and C3H mammary tumor cells to determine optimal chamber loading densities, drug dosing, and harvesting times. Different concentrations of L1210 cells were loaded into chambers and examined at intervals from 2 to 7 days. An initial loading density of 5×10^6 cells/ml provided an adequate density of cells for examination at early times without excessively dense multilayered growth at later stages. Suspensions of C3H mammary and human tumor cells loaded at this initial concentration (5×10^6 cells/ml) also gave acceptable membrane cell densities at times of interest. In all subsequent experiments this initial loading density was used.

Tests with L1210 cells showed that a 2-day period of culture produced a healthy, actively growing monolayer of attached cells as evidenced by numerous mitotic figures. With C3H tumor cells a 3-day growth period was necessary to produce equivalent levels of growth. Drug treatment was initiated, therefore, 2 days after chamber implantation for assays using L1210 leukemia and 3 days after chamber implantation for C3H mammary tumor.

Duplicate chambers were removed from control and drug-treated animals at 12 hourly intervals after dosing with the drugs listed in table 1. Cell numbers on the membranes were compared. All membranes were fully examined at low magnification and the cells in a constant number of high power fields (× 400) were counted and the totals averaged. Cells showing morphologic changes (karyorrhexis, anisocytosis, pyknosis, chromatolysis, and autolysis), which are regarded by cytologists as presumptive evidence of imminent cell death, were excluded from the counts. Harvesting 48 hours after the end of drug dosing provided the best contrast between drug-treated and control cell populations. The counts observed 48 hours after dosing are provided in table 1. When assaying drug sensitivity of the L1210/63878 subline, chambers were examined only at 48 hours after dosing.[12]

L1210 Assays

C3H/HeJ female × DBA_2/J male F_1 hybrids weighing 18.5–22.5 g were inoculated ip with 10^5 L1210 cells on Day 0. Drugs were given ip starting 24 hours after tumor implantation. There were six animals per dose level and one control group for every five test groups. Mean survival times of control groups ranged from 9.5 to 10.5 days. Drugs were examined at a series of concentrations (separated by 0.18 log dose intervals) ranging from clearly toxic levels to inactive levels. Certain agents proved nontoxic and these were tested at the maximum limit imposed by practical considerations (500 mg/kg). Animal deaths were recorded twice daily. Except for cyclophosphamide the life extensions recorded (table 1) are those obtained at the optimum dose—the dose used in the diffusion chamber assays. Cyclophosphamide was given at the level used in this laboratory for priming purposes (150 mg/kg).

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[12] Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are included in the List of Compounds at the end of the text. Compounds were generously supplied by the manufacturers.

C3H Mammary Tumor Assays

Spontaneous mammary tumors in elderly C3H/HeJ breeding females were detected during the course of routine handling. Tumors were dissected aseptically and minced with fine scissors. Trocar implants of tumor fragments measuring approximately 1 mm in diameter were made in the axillary region of C3H/HeJ female mice weighing 18–25 g. As a matter of policy transplants were not carried past the first generation. Only mice that had received implants from a single tumor were used in any one group of tests. When tumor implants had grown, mice with regularly shaped tumors (between 6 × 6 and 8 × 8 mm in size) were selected and randomized into groups of eight animals; two such groups were retained as controls. Tumor diameters were measured twice weekly along two perpendicular axes with vernier calipers. Tumor volumes were calculated using the formula of a prolate sphere, $4/3\ \pi ab^2$, where a and b are the major and minor semiaxes respectively (14).

To best utilize tumor-bearing mice considerable toxicity data were obtained for normal C3H mice. From the many drug studies we examined it was possible to estimate an approximate LD10 for the projected dosing schedules. A series of logarithmically spaced doses close to this approximate LD10 were then administered to groups of C3H mice (ten mice for each dose level) using the dosage regimen required. Percentage of lethality was plotted on log-probit paper in the usual way and a more accurate LD10 was then derived (15). Doses in the experimental tumor treatment groups were set 0.07 log dose below the LD10 obtained; with the experimental variations involved these doses then represent 0.7–1.0 LD10 for the dosing schedule. In the case of cyclophosphamide, considerable previous experience allowed a single priming dose of 150 mg/kg to be selected. In the absence of more definitive data the treatment schedules adopted have been those considered highly effective in treating L1210 leukemia in mice (16).

Agents

Most of the agents we used were supplied by pharmaceutical houses and are the same samples used in the clinic. Water-soluble drugs were administered in 0.9% saline solution, and insoluble drugs were administered as homogenized suspensions in 0.9% saline containing methylcellulose (0.5%) and benzyl alcohol (0.9%). In all cases, drugs were injected ip using a standard volume of 0.01 ml/g of animal weight.

RESULTS

Assaying for intrinsic drug sensitivity of L1210 cells was carried out using C3H mice as substitute hosts; this strain does not normally support growth of this tumor. There appeared to be little evidence of immunologic effect against L1210 growing in chambers in C3H mice. If C3H hosts were pre-immunized with 10^5 L1210 cells ip weekly for 3 weeks then L1210 cells in implanted millipore diffusion chambers appeared to grow as well in pre-immunized animals as did corresponding controls in nonimmunized animals.

To score a statistically significant response in leukemic animals (increase in life survival >25%) a drug must, for the dose and schedule employed, produce slightly more than a 1 log (90%) kill in the leukemic cell population (17). Correspondingly, the acceptance point for a positive drug-tumor matching response with L1210 in diffusion chambers should then be a greater than 90% reduction in cell density or observation of the morphologic changes that precede death in greater than 90% of the cells. Using these criteria, the diffusion chamber technique enabled us to predict those agents which would produce statistically significant extensions of life in the usual ip implanted L1210 screening test (18).

The results of this study are presented in table 1; the diffusion chamber matching results are in columns 4 and 5 and the life extensions obtained in ip implanted L1210 using the same drug doses as in the matching tests are in column 6.

L1210/63878 (a cytosine arabinoside-resistant variant of L1210 leukemia) was available from other studies. Drug-tumor sensitivity matching of this variant clearly demonstrated the drug resistance (table 1, columns 7 and 8; fig 1). From the chamber tests and screening data (table 1, columns 7–9)

there appeared to be no cross resistance of the tumor variant to the other agents employed.

The pattern of intrinsic drug sensitivity of the C3H spontaneous mammary tumor was determined essentially as described for L1210 leukemia except that nonsyngeneic DBA_2/J mice were used as substitute hosts.

It is known that inflicting a greater than 90% tumor cell kill on an experimental solid tumor need not significantly reduce tumor dimensions (19). It therefore appeared that the endpoint used when drug matching to L1210 leukemia (> 90% tumor cells affected) could be realistically adopted for drug matching experimental solid tumors. The results of the drug-tumor sensitivity matching for the C3H tumor, using this criterion, are recorded in table 1 (columns 10 and 11). Of those drugs which were scored as positive, vinblastine (14, 20), cyclophosphamide (14, 21), 6-mercaptopurine (14), and daunorubicin (22) have been previously shown to slow the growth of this tumor.

Animals with first-generation transplants of the spontaneous C3H mammary carcinoma were treated with the agents selected by the matching procedure. Plots of tumor volume against time are presented in figures 2 and 3. By selection, an appropriate time could be chosen when the mean tumor volume of any drug-treated group was significantly different from the control tumor volume ($P<0.05$, "Student's t" test). It is clear from the figures that the percentage of tumor volume inhibition (T/C) varies according to the time selected for evaluation. It has been stressed (23) that the percentage of tumor inhibition depends on the time of evaluation. No statistically significant reduction of initial tumor volume was seen in this group of experiments; it appeared rather that the tumor volume increase of the drug-treated groups was considerably slowed.

It is clear that all drugs selected by sensitivity matching, using the diffusion chamber technique, are capable of significantly affecting the growth of this tumor. Thus, the drug-tumor sensitivity matching scheme selected no false positive agents against either the rapidly growing, high growth fraction, ascitic L1210 leukemia or the slow-growing, low growth fraction, solid C3H spontaneous mammary tumor. There is not sufficient information available to eliminate the possibility that there may be false negatives in the agents discarded on the basis of the diffusion chamber experiments with the C3H tumor, although many of the discarded agents have been examined in this system (14,21,24–28).

Two drugs (cytosine arabinoside and progesterone) which, to our knowledge, had not been tested against the C3H tumor have now been screened (fig 4), and it was shown that they did not significantly affect tumor growth with the drug doses and schedules used. In this same group of experiments (fig 4) it was found that, with the doses employed, 6-mercaptopurine given daily on Days 0–9 appeared to produce less tumor volume inhibition than when given on an every-4-day schedule (fig 2).

The observed resistance to standard chemotherapy (21,24–28) of the slow-growing C3H mammary carcinoma of viral etiology provides a considerable challenge to the experimental chemotherapist. Accordingly, we have examined multiple-agent treatment of this tumor using the drugs selected by the diffusion chamber matching procedure. Even with the relatively small number of drugs selected by the matching procedure there are many ways in which these agents could be tried in combination. Accordingly, we examined methods of reducing the possible number of permutations. NSC-150440 was first eliminated from further consideration and only those drugs presently used in the clinic were retained. For preliminary studies it was decided to use only one of the vinca alkaloids; vincristine was selected. The desirability of starting treatment of a low growth fraction tumor with a cell-cycle-stage-nonspecific agent has been convincingly demonstrated (19,29,30). Accordingly, from the remaining agents under consideration, cyclophosphamide could be chosen as the first member for projected cyclic schedules. A study was made of the effect produced by administering the remaining drugs after a priming dose of cyclophosphamide (fig 5). It was hoped that the information gained from this experiment would allow a more intelligent selection of agents for an effective cyclic treatment and possibly provide information that would help determine the order of drug application. Note

FIGURES 1*a*–1*f*.—Photomicrographs of in vivo cultured animal tumor cells on millipore membranes removed from diffusion chambers. All membranes were harvested at an equivalent time, 48 hrs after the end of drug dosing.

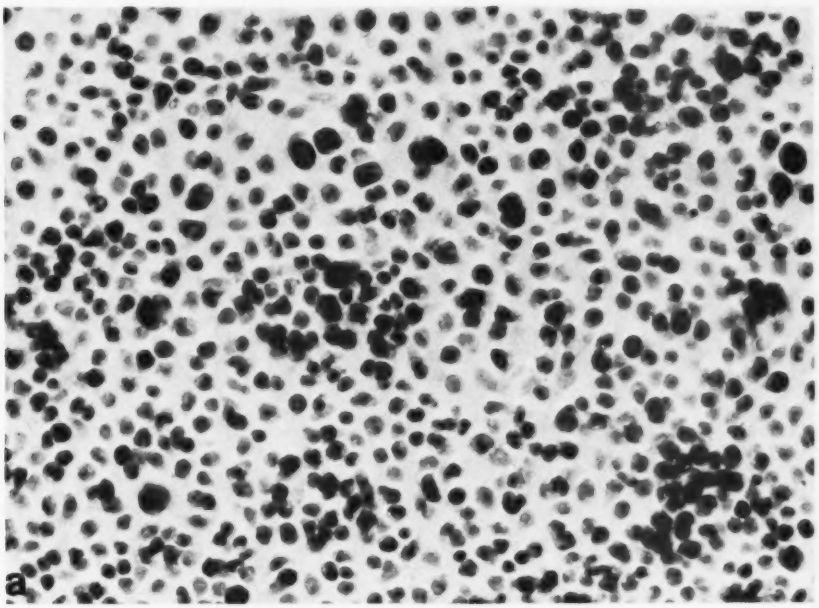

FIGURE 1*a*.—Parental L1210 strain (control).

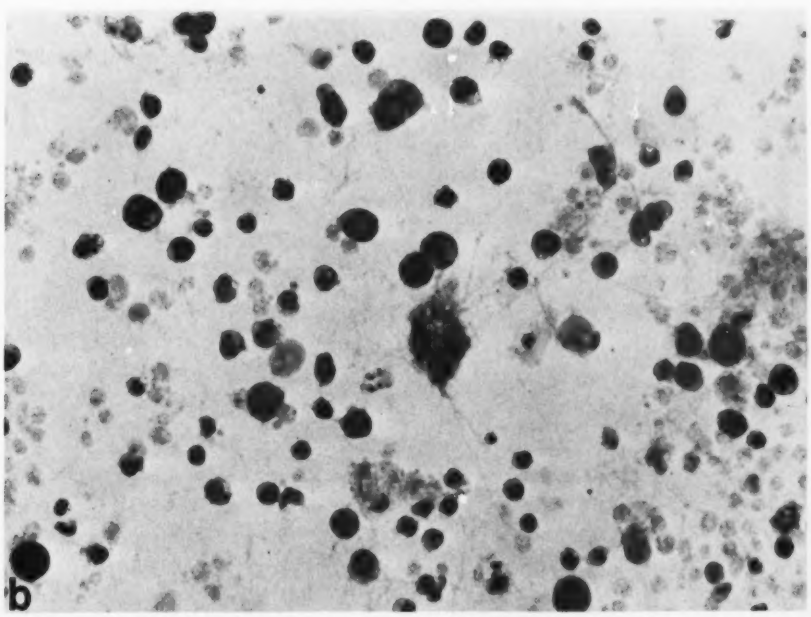

FIGURE 1*b*.—Parental L1210 strain; host animal treated with cytosine arabinoside (22 mg/kg/dose, q3h × 8).

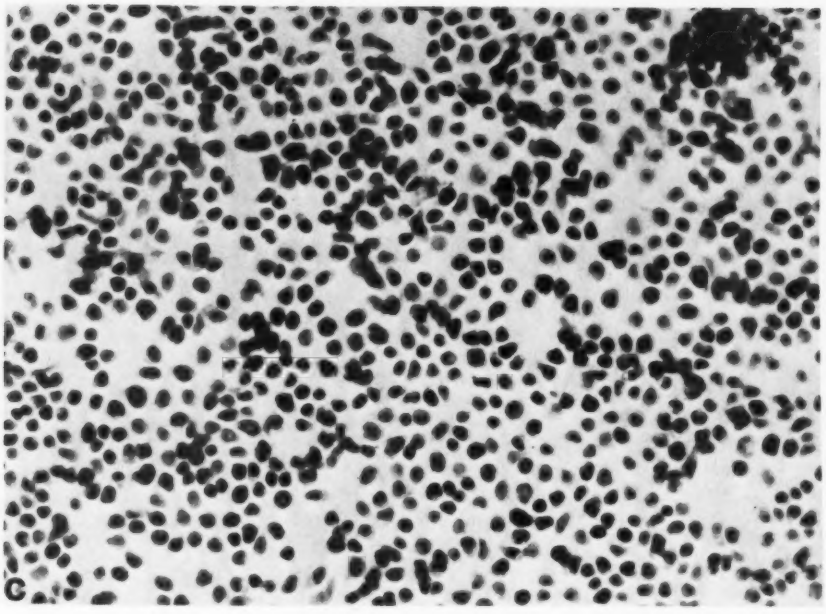

FIGURE 1*c*.—Resistant L1210/63878; host animal treated with cytosine arabinoside (22 mg/kg/dose, q3h × 8).

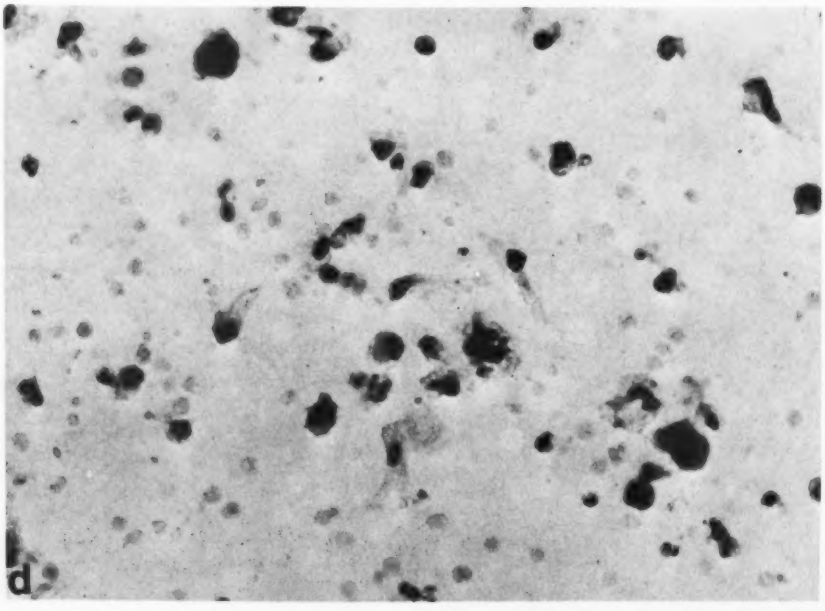

FIGURE 1*d*.—Parental L1210 strain; host animal treated with cyclophosphamide (150 mg/kg).

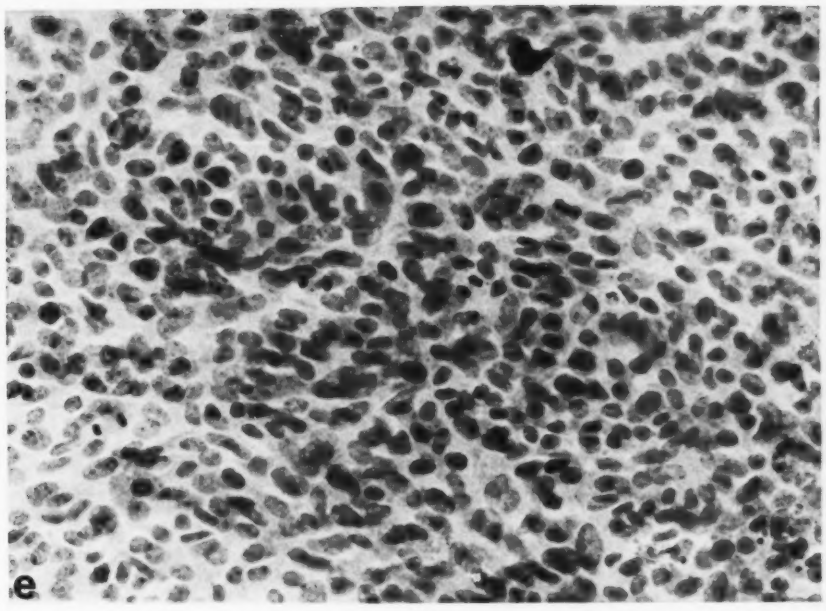

FIGURE 1*e*.—C3H spontaneous mammary carcinoma (control).

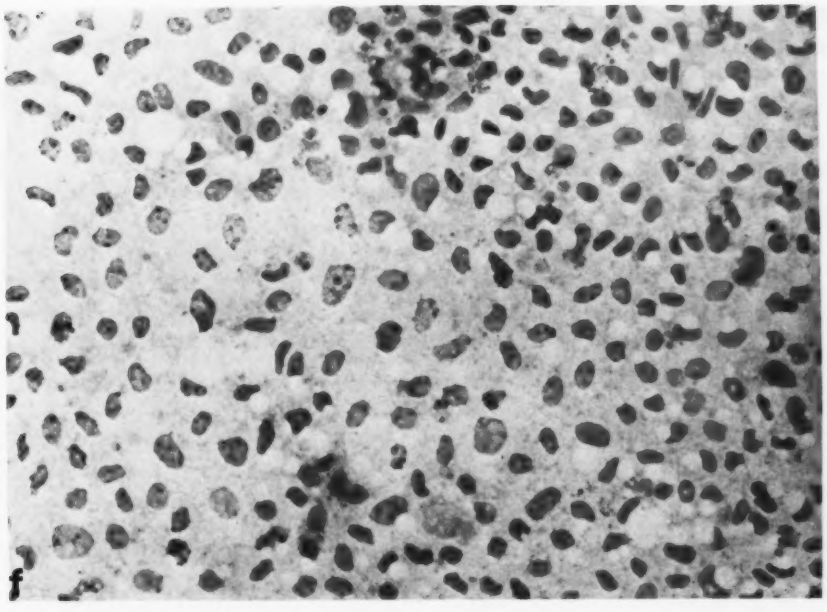

FIGURE 1*f*.—C3H spontaneous mammary carcinoma; host animal treated with hydroxyurea (100 mg/kg/dose, q3h × 8). Note that the majority of the cells remaining show evidence of drug damage (irregular shapes, vacuolation, chromatin disturbance) and were not included in counts of undamaged cells.

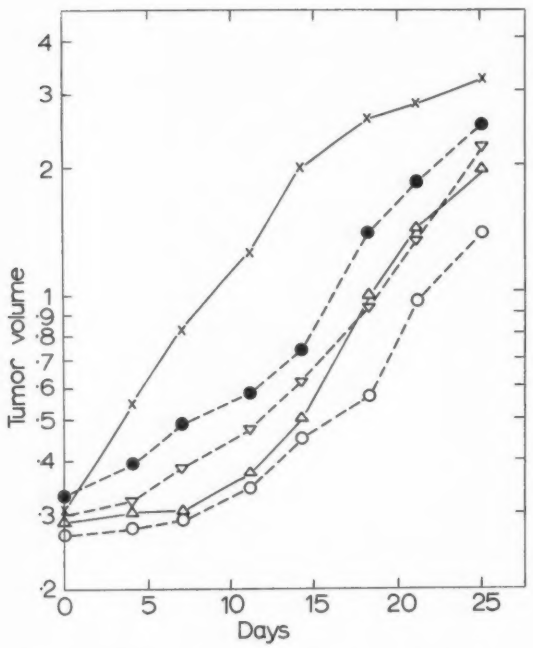

FIGURE 2.—Effect of drug treatments on tumor volume (cm^3) increase of first-generation transplants of C3H spontaneous mammary tumors. All drugs were administered on Days 0, 3, 7, and 11.

×———× = Untreated controls.
●·····● = Actinomycin D: 0.31 mg/kg/day.
▽·····▽ = Vinblastine: 1.25 mg/kg/day.
△———△ = Hydroxyurea: 75 mg/kg/dose, q3h × 8.
○·····○ = 6-Mercaptopurine: 208 mg/kg/day.

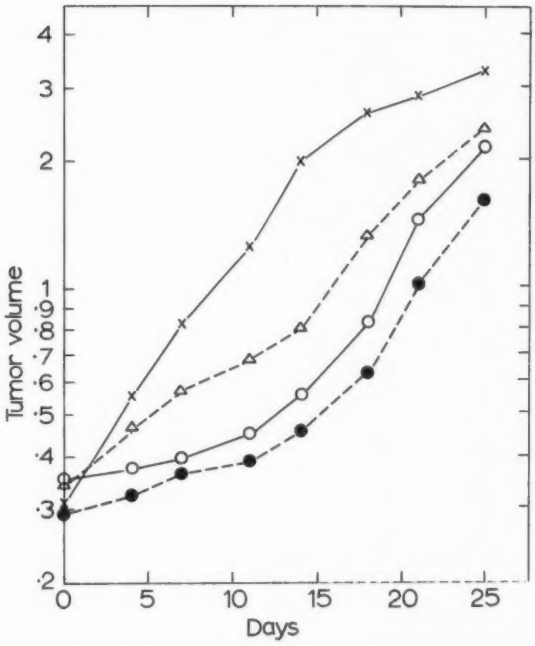

FIGURE 3.—Effect of drug treatments on tumor volume (cm^3) increase of first-generation transplants of C3H spontaneous mammary tumors. All drugs were administered on Days 0, 3, 7, and 11.

×———× = Untreated controls.
△·····△ = Daunorubicin: 2.2 mg/kg/day.
○———○ = Vincristine: 0.83 mg/kg/day.
●·····● = NSC-150440: 21 mg/kg/day.

that the doses used in these experiments are those used in gaining the information depicted in figures 1 and 2. Higher single doses of the second agents in these treatments (fig 5) could have been used but we were interested in the effect that would be produced by a dose of drug which could be expected to be tolerated during more prolonged therapy.

At the doses and schedules involved it appeared that both actinomycin D and daunorubicin used singly (figs 2 and 3) or after cyclophosphamide (fig 5) were not as tumor inhibitory as the other agents used. These two agents were therefore not considered further as components of cyclic schedules. However, after the completion of the experimental work reported in this paper, Lenaz and Di Marco (31) suggested that the reason some authors were obtaining poor results in the treatment of solid tumors with daunorubicin was because of the low drug levels reaching the tumor after ip administration. In their own experiments (22) using intravenous drug administration, these authors obtained excellent tumor inhibitory effects.

The remaining agents under consideration, cyclophosphamide, hydroxyurea, vincristine, and 6-mercaptopurine, were applied in the order stated to tumor-carrying animals. The results of a single cycle of treatment are presented in figure 6. It appeared that there

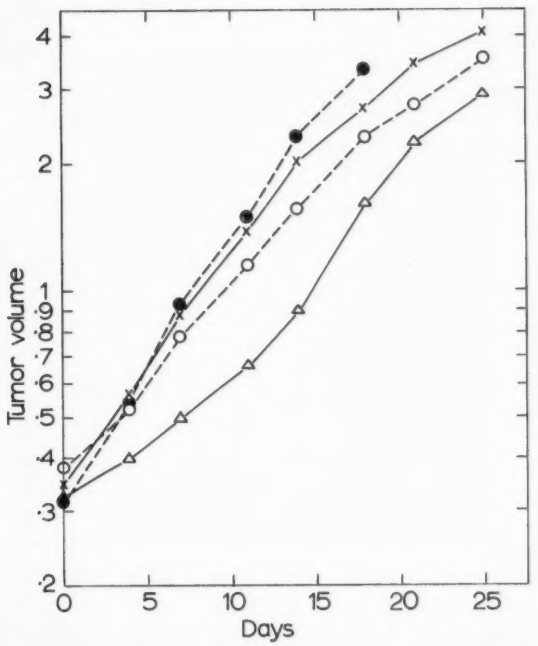

FIGURE 4.—Effect of drug treatments on tumor volume (cm^3) increase of first-generation transplants of C3H spontaneous mammary tumors.

×———× = Untreated controls.
●- - - - - - - - - - - - ● = Progesterone: 500 mg/kg/day, qd Days 0–9.
○- - - - - - - - - - - - ○ = Cytosine arabinoside: 16 mg/kg/dose, q3h × 8 on Days 0, 3, 7, and 11.
△- - - - - - - - - - - - △ = 6-Mercaptopurine: 71 mg/kg/day, qd Days 0–9.

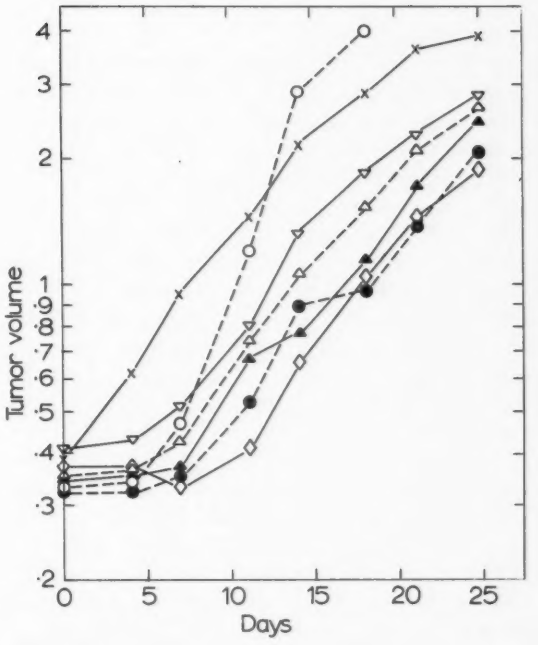

FIGURE 5.—Effect of drug treatments on tumor volume (cm^3) increase of first-generation transplants of C3H spontaneous mammary tumors. All drug-treated animals received a priming dose of cyclophosphamide (150 mg/kg on Day 0).

○- - - - - - - - - - - - ○ = Priming dose alone.

On Day 3 additional treatments were given as follows:

▽———▽ = Actinomycin D: 0.31 mg/kg.
△- - - - - - - - - - - - △ = Daunorubicin: 2.2 mg/kg.
▲———▲ = Vincristine: 0.83 mg/kg.
●- - - - - - - - - - - - ● = Hydroxyurea: 75 mg/kg/dose, q3h × 8.
◇———◇ = 6-Mercaptopurine: 208 mg/kg.

was no increase in tumor volume during treatment. In later experiments two cycles of treatment were applied and, again, tumors which were moderate in size (0.3 cm^3) at the start of treatment did not increase in volume (fig 6). Measured from the first day of drug administration the mean survival time recorded in this later experiment (T/C, 210%) appears large compared to those recorded by others who have treated this tumor (14,20–22,24–28). While the cyclic therapy (fig 6) appears superior to other treatments (figs 2–5), it should be recalled that full dose- and schedule-dependency studies have not been carried out with the single agents (figs 2 and 3) and no rigorous conclusion can be drawn without additional data.

Attempts to administer more than two courses of the cyclic treatment to either normal mice or mice with C3H tumors inevitably caused toxic deaths. In our total experience there were five deaths among 48 animals during application of a third cycle, 11 deaths among 43 animals during a fourth cycle, 21 deaths among 32 animals during a fifth cycle, and all remaining mice but one died during an attempted sixth cycle. These figures show considerable cumulative toxicity; it would ap-

pear advisable to allow a longer (> 4-day) recovery period after the application of two cycles of treatment.

Animals bearing larger tumors (1.2 cm^3) were treated with two courses of cyclic therapy, but in these experiments tumors continued to progress (fig 7). The growth kinetics of the C3H tumor have been shown to be Gompertzian (30) and the implications of such growth characteristics have been clearly stated (19, 29,30). This further example of change in drug response with tumor size (19,30) prompted an evaluation of the cyclic therapy given when initial tumor size was smaller. When treatment was initiated 24 hours after tumor implantation three animals from a group of eight failed to develop tumor within twice the median survival time of the untreated control group. These latter results and a confirmatory experiment are summarized in table 2. In these experiments it does appear that cyclic treatment has produced better results than have been previously obtained with this tumor (14,20–22, 24–28).

DISCUSSION

It is not surprising that we obtained a good correlation between the results of the diffusion chamber drug-tumor matching procedure and the tumor growth inhibition studies using L1210 leukemia. In both the diffusion chamber and screening tests drugs are applied in close proximity to the tumor cells. The imposition of a millipore membrane between the leukemic cells and the administered drugs appears not to prevent the agents from reaching tumor cells in effective concentrations. The diffusion chamber procedure does, however, allow a ready visualization of drug effectiveness.

In retrospect, it appears that we were fortunate to obtain a correlation between the results from the matching procedure and the growth inhibition studies using the slow-growing, low growth fraction (30), C3H mammary carcinoma. There are several reasons for expecting that the sensitivity of ip cultured tumor cells to ip administered drug may not predict drug sensitivity of the parent tumor in its host of origin:

(1) *Cytokinetic factors.*—There are only low levels of population pressures acting on chamber-cultured cells which consequently may have a higher growth fraction than cells of the parent tumor. If such cultured cells appeared sensitive to a cell-cycle-stage-specific agent it need not follow that the agent would significantly inhibit the low growth fraction parent tumor. Further, when assaying for drug sensitivity in the diffusion chamber tests we have required that greater than 90% of the cultured cells be drug affected for a positive response. With cell-cycle-stage-specific agents, the number of cultured cells killed will be a function of (*a*) the growth fraction of the

TABLE 2.—Cyclic drug therapy for first-generation transplants of spontaneous mammary tumors in female C3H mice

Group	Average survival time (days ± SE)*	No. of animals dying with tumor/ total treated	No. of survivors without tumor†/ total treated
Expt A			
Control	72.4 ± 4.8	16/16	0/16
Treated‡	128.0 ± 6.0§	5/8	3/8
Expt B			
Control	76.7 ± 4.9	24/24	0/24
Treated‡	132.5 ± 6.5§	11/16	5/16

*Long-term survivors excluded.

†150 days after the start of experiment; about twice the mean survival time of untreated controls.

‡Cyclophosphamide, Days 0 and 15, 150 mg/kg; hydroxyurea, Days 3 and 19, 75 mg/kg/ dose, q3h × 8; vincristine, Days 7 and 23, 0.83 mg/kg; 6-mercaptopurine, Days 11 and 27, 208 mg/kg.

§Significantly different from corresponding control ($P < 0.001$).

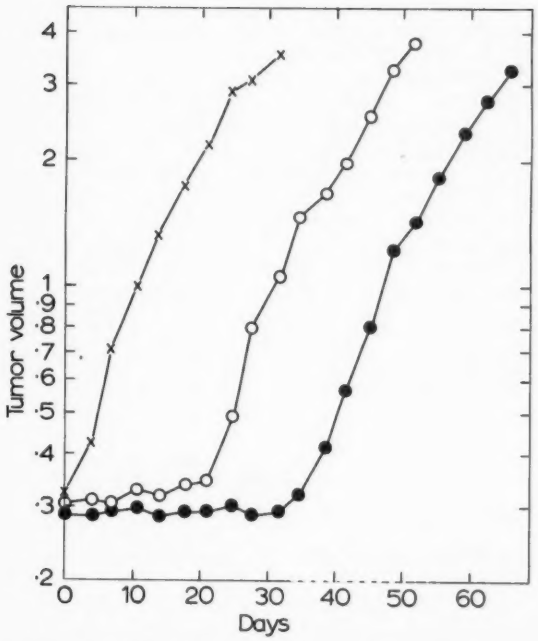

FIGURE 6.—Effect of drug treatments on tumor volume (cm^3) increase of first-generation transplants of C3H spontaneous mammary tumors.

●———● = Cyclophosphamide (150 mg/kg on Days 0 and 15), hydroxyurea (75 mg/kg/dose, q3h × 8 on Days 3 and 19), vincristine (0.83 mg/kg on Days 7 and 23), 6-mercaptopurine (208 mg/kg on Days 11 and 27).

×———× = Controls associated with the above group.

○———○ = Animals in this group received 1 cycle of treatment, that is, the first doses of the above sequence, those administered on Days 0, 3, 7, and 11.

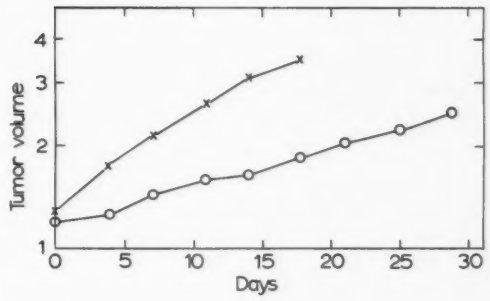

FIGURE 7.—Effect of drug treatments on tumor volume (cm^3) increase of first-generation transplants of C3H spontaneous mammary tumors.

×———× = Untreated controls.

○———○ = Cyclophosphamide (150 mg/kg on Days 0 and 15), hydroxyurea (75 mg/kg/dose, q3h × 8 on Days 3 and 19), vincristine (0.83 mg/kg on Days 7 and 23), 6-mercaptopurine (208 mg/kg on Days 11 and 27).

cultured cells, (*b*) the cell cycle time of those cells, and (*c*) the time for which effective drug concentrations persist in the substitute host (1). Several situations can be foreseen that would result in drug-sensitive populations providing false negative responses because the necessary 90% tumor cell kill was not reached.

(2) *Pharmacologic factors.*—Ip administration of drug to ip chamber-cultured tumor cells applies a high concentration of drug directly to the target cells. In the autochthonous tumor host the concentration of agent reaching target cells depends on the anatomic location of those cells, the route of drug administration, the physiologic disposition of the agent, etc.

(3) *Biochemical factors.*—The comparative biochemistries of the substitute host used in the diffusion chamber matching procedure and the original tumor host will influence the levels of enzymes and antagonistic metabolites to which an agent is exposed. The ability of an individual host to perform a necessary enzymatic activation step (eg, cyclophosphamide) will determine if treatment with that drug can be effective in that host. High host levels of certain enzymes (eg, cytidine aminohydrolase) can prevent effective blood concentrations of agent (cytosine arabinoside) from being maintained for a sufficient time (32). Similarly, high host blood levels of deoxycytidine can antagonize the antitumor action of cytosine arabinoside (8–10) even though tumor cells are intrinsically drug sensitive.

It is clear that the results from diffusion chamber drug-tumor matching experiments

should be interpreted with caution. Negative results might be incorrect. In contrast, a positive response provides definite evidence that the tumor cells are intrinsically drug sensitive. It may, therefore, be possible, by using such matching tests, to select from a panel of drugs a group of agents which have a better chance of producing favorable results than a group selected randomly. Such selection could be extremely important when attempting to obtain the best possible results using multiple-agent therapy.

Effective means of assaying intrinsic drug sensitivities of tumor cells could provide a valuable first step towards prediction of drug responsiveness of a tumor. It will be necessary to have considerably more knowledge of the factors operating at the biochemical, cytokinetic, and pharmacologic levels before reliable prediction of tumor response will be possible.

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LIST OF COMPOUNDS

Actinomycin D: NSC-3053

Bleomycin: NSC-125066

Busulfan: NSC-750; CAS reg. No. 55-98-1; 1,4-butanediol, dimethanesulfonate

Chlorambucil: NSC-3088; CAS reg. No. 305-03-3; butyric acid, 4-[*p*-[bis(2-chloroethyl)amino]phenyl]-

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate

Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride

Dexamethasone: NSC-34521; CAS reg. No. 50-02-2; pregna-1,4-diene-3,20-dione, 9-fluoro-11β,17,21-trihydroxy-16α-methyl-

Daunorubicin: NSC-83142; CAS reg. No. 23541-50-6; daunomycin (NSC-82151)

Diethylstilbestrol: NSC-3070; 4,4′-stilbenediol, α,α′-diethyl-; stilbestrol

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU

Fluoxymesterone: NSC-12165; CAS reg. No. 76-43-7; androst-4-en-3-one, 9-fluoro-11β,17β-dihydroxy-17-methyl-

Hydroxyurea: NSC-32065; CAS reg. No. 127-07-1

Medroxyprogesterone: NSC-26386; pregn-4-ene-3,20-dione, 17-hydroxy-6α-methyl-, acetate

Malengestrol acetate: NSC-70968; CAS reg. No. 2919-66-6; pregna-4,6-diene-3,20-dione, 17-hydroxy-6-methyl-16-methylene-, acetate

Mechlorethamine: NSC-762; CAS reg. No. 55-86-7; diethylamine, 2,2′-dichloro-*N*-methyl-, hydrochloride; nitrogen mustard

Melphalan: NSC-8806; CAS reg. No. 3223-07-2; alanine, 3-[*p*-[bis(2-chloroethyl)amino]phenyl]-, monohydrochloride, L-

6-Mercaptopurine: NSC-755; CAS reg. No. 6112-76-1; purine-6-thiol, monohydrate; 6-MP

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]-methylamino]benzoyl]-

Mithramycin: NSC-24559; CAS reg. No. 18378-89-7

Mitomycin C: NSC-26980; CAS reg. No. 50-07-7

Nitromin: NSC-10107; diethylamine, 2,2′-dichloro-*N*-methyl-, *N*-oxide, compd. with HCl (1:1)

NSC-29215: CAS reg. No. 68-76-8; 2,3,5-tris(1-aziridinyl)-*p*-benzoquinone; Trenimon

NSC-122819: 4′-demethylepipodophyllotoxin, 9-(4,6-*O*-2-thenylidene-β-D-glucopyranoside); VM-26

NSC-150440: methanesulfon-*m*-anisidide, 4′-[(4-methyl-9-acridinyl)amino]-, methanesulfonate

Prednisone: NSC-10023; CAS reg. No. 53-03-2; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-

Procarbazine: NSC-77213; CAS reg. No. 491-88-3; *p*-toluamide, *N*-isopropyl-α-(2-methylhydrazino)-, monohydrochloride

Progesterone: NSC-9704; CAS reg. No. 57-83-0; pregna-4-ene-3,20-dione

Vinblastine: NSC-49842; CAS reg. No. 6449-03-2; vincaleukoblastine, sulfate (1:1), monohydrate

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

Clinical Trial of 5-Hydroxypicolinaldehyde Thiosemicarbazone (5-HP; NSC-107392), With Special Reference to Its Iron-Chelating Properties [1,2,3]

Irwin H. Krakoff, Erlinda Etcubanas, Charlotte Tan, Klaus Mayer, Victor Bethune, and Joseph H. Burchenal [4,5]

SUMMARY

5-HP is a substituted pyridine compound which inhibits ribonucleoside diphosphate reductase by the chelation of iron. It was given intravenously to 30 patients with leukemia or various solid tumors. The major side effects were severe nausea, vomiting, fever, chills, diaphoresis, abdominal pain, hypertension, hypotension, anemia, and bone marrow depression. Marked hemolysis, iron chelation, and urinary excretion of iron occurred in association with 5-HP administration. Three patients with acute leukemia achieved relative decreases in the percentage of blast cells in the bone marrow; these were of brief duration and were not clinically useful.

[Cancer Chemother Rep 58:207–212, 1974]

The carcinostatic activity in transplanted animal tumors of a group of heterocyclic aldehyde thiosemicarbazones has been reported in the past (1–6). The spectrum of animal systems in which these agents have shown antitumor activity includes L1210 and L5178Y leukemias, Lewis lung carcinoma, Adenocarcinoma 755, and Ehrlich ascites carcinoma. Antiviral activity has also been observed (7, 8). There was significant correlation between the antitumor activity of the compounds and their metal-chelating properties (2–4,9–11). These compounds are thought to exert their antitumor activity by inhibition of DNA synthesis at the level of the enzyme, ribonucleoside diphosphate reductase, presumably by chelation of the iron moiety of the enzyme molecule (10–16). This is the same mechanism by which hydroxyurea [6] exerts its cytotoxic and therapeutic activity (14,17). It was demonstrated that by substitutions within the ring system, it was possible to synthesize active thiosemicarbazones possessing variable degrees of activity and toxicity (4,5,18).

Among these derivatives, the hydroxy-substituted pyridine thiosemicarbazones were found to be the most soluble and easily formulated (12,19). On this basis, 5-hydroxypicolinaldehyde thiosemicarbazone (5-HP) was chosen as the most appropriate member of this group of compounds for clinical trials.

In animals, French and Blanz (4) found that by the intraperitoneal (ip) route, 5-HP was active against L1210 and L5178Y leukemias

[1] Received July 20, 1972; revised Oct 4, 1973; accepted Nov 27, 1973.

[2] Supported in part by grants CA-08748 and CA-05826 from the National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3] 5-HP: CAS reg. No. 19494-89-4; 5-hydroxy-2-formylpyridine thiosemicarbazone. Supplied by Cancer Therapy Evaluation, Division of Cancer Treatment, NCI.

[4] Medical Oncology (Dr. Krakoff) and Hematology (Drs. Mayer and Burchenal) Services, Department of Medicine, and the Departments of Pediatrics (Drs. Etcubanas and Tan) and Biochemistry (Dr. Bethune), Memorial Sloan-Kettering Cancer Center, New York, NY.

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[6] Hydroxyurea: NSC–32065; CAS reg. No. 127-07-1.

and Ehrlich ascites carcinoma. The single-dose LD50 (ip) was 2200 mg/kg in BDF_1 female mice. On autopsy, BDF_1 female mice that had received ip injections of 5-HP showed variations in the size of red blood cells and erythroid hyperplasia in the bone marrow.

Previous reports have reviewed some of the clinical effects of this agent (16,20,21).

MATERIALS AND METHODS

5-HP is a yellow powder supplied in sterile vials, each containing 500 mg. It was dissolved in physiologic saline at a concentration of 50 mg/ml. The solution was stable for 24 hours.

5-HP was given to 30 patients (adults and children), 20 with leukemia and ten with a variety of solid tumors, all of whom had previously received other chemotherapeutic agents but had recovered from toxicity induced by this prior therapy. The trials were carried out on an inpatient or outpatient basis, depending on each patient's clinical status.

The drug was given intravenously (iv) daily in (*a*) single or multiple infusions lasting for 1–12 hours, (*b*) single or multiple injections by iv push, or (*c*) 24-hour infusions. Those treated by 24-hour infusions tolerated the treatment best, with the least nausea and vomiting. Based on toxicologic studies in dogs, an initial dose of 0.5 mg/kg/day was given (one tenth the minimum toxic dose in dogs). The daily dose was increased stepwise in individual patients, depending on tolerance, up to a maximum of 76 mg/kg/day. After the demonstration of "tolerable" toxicity in the first few patients who received 0.5 mg/kg/day, subsequent patients were treated with larger doses up to a maximum starting dose of 30 mg/kg/day. Administration of 5-HP was continued for a maximum of 49 days and to a maximum total dose of 1653 mg/kg. An "adequate" trial has been arbitrarily defined (*see* below) on the basis of hematologic toxicity; 20 patients are considered to have received "adequate" trials.

The response to treatment was evaluated by the usual parameters: signs and symptoms referrable to the disease, measurable palpable organs or tumors, biochemical alterations, radiologic evidence of disease, and hematologic abnormalities. These were followed periodically during the course of treatment. Evidence of clinical or hematologic toxicity was evaluated prior to each dose. Routine urinalysis and biochemical tests for liver and renal function were done at weekly intervals.

The response to treatment in children with leukemia was evaluated according to the "Criteria for Evaluating Chemotherapy in Acute Leukemia" published by the Midwest Cooperative Chemotherapy Study Group (22).

Serum and urinary iron levels determined by a colorimetric method (23) yielded spuriously low values due to chelation of iron by 5-HP. Therefore, the values reported in this study were measured by atomic absorption spectrometry (24).

5-HP was assayed by the method of Tomchick and Mead (25). After extraction from plasma or urine with ethyl acetate, the resulting extract was re-extracted with sodium carbonate solution. It was then diluted with distilled water and the 5-HP content was read at 347μ in a spectrophotometer.

RESULTS

Toxicity

Severe nausea and vomiting were the most commonly observed side effects in the majority of the patients. They occurred as early as 10 minutes after injection of the drug. Within 2–4 hours after drug administration, one or more of the following also developed: fever, tachycardia, diarrhea, abdominal pain, hypertension, hypotension, chills, leg cramps, or diaphoresis. These usually lasted for 3–6 hours (table 1). At the same time, the patients excreted green-brown urine, which was shown to be due to the excretion of iron complexed with 5-HP. An identical green color was noted when colorless aqueous solutions of 5-HP and an iron salt were mixed in vitro. The urine became clear within 24 hours. Occasionally, treatment had to be interrupted temporarily because of hypertension, hypotension, or tachycardia. Four patients had transient arrhythmias detected by electrocardiogram.

The hematologic effects are shown in table 2. Anemia, leukopenia, thrombocytopenia, reticulocytosis, and megaloblastic changes in the marrow were seen both in patients with solid tumors and patients with leukemia. Although difficult to evaluate in patients with acute leu-

TABLE 1.—Side effects in 30 patients given 5-HP

Side effect	No. of patients
Nausea, vomiting	19
Fever	13
Green urine	9
Tachycardia	8
Diarrhea	6
Abdominal pain	5
Hypertension	5
Hypotension	5
EKG changes	4
Chills	3
Leg cramps	2
Diaphoresis	2

kemia, the hematologic effects observed in patients with solid tumors occurred against a stable hematopoietic background and could be readily interpreted as drug effect. The average drop in hemoglobin levels was 4 g/100 ml with a range of 2–6 g/100 ml.

The maximum depression of peripheral blood cell counts occurred within 2 weeks after the start of therapy, and recovery occurred 7–10 days after the cessation of therapy.

During the course of the study, it was observed that 5-HP formed a strong complex with iron. Assays of synthetic 5-HP:iron mixtures by the procedure described previously resulted in poor recoveries of 5-HP. This indicated that the 5-HP:iron complex was firmly bound and the ethyl acetate extractant was unable to remove quantitatively the 5-HP present. Attempts to displace the 5-HP from the iron complex with EDTA failed to yield satisfactory recoveries. Thus, neither iron nor 5-HP levels could be determined colorimetrically in either serum or urine. However, iron levels could be determined accurately and reproducibly by atomic absorption spectrometry and all of the values quoted in these studies were obtained in that manner.

TABLE 2.—Hematologic toxicity of 5-HP

Hematologic effect	Patients with— Leukemia (total No., 20)	Solid tumors (total No., 10)
Anemia (hemoglobin decrease ≥2 g/100 ml)	16	6
Leukopenia (<4000 cells/mm³)	8	5
Thrombocytopenia (<100,000 platelets/mm³)	8	3
Reticulocytosis (>1%)	14	5
Megaloblastosis	9	3

The influence of 5-HP on iron homeostasis in a clinical experiment is shown in figure 1. This patient, a 64-year-old man with recurrent inoperable carcinoma of the floor of the mouth, was given 5-HP daily for 3 weeks. Periodic sampling and measurement during the first 24 hours after the rapid injection of a dose of 10 mg/kg revealed a prompt and marked increase in urinary iron and iron-binding capacity. The urinary iron-binding capacity, normally very low in the absence of proteinuria (as was true in this case), is considered to be due to the presence in the urine of available or uncomplexed 5-HP. Both urinary iron and iron-binding capacity reached their peaks during the second hour after injection of 5-HP and declined gradually during the next 22 hours. In the next 20 days, during which 5-HP was administered daily except for a single day's hiatus, larger amounts than normal of iron were consistently excreted. This was accompanied by a gradual decrease in the hemoglobin level, requiring the transfusion of packed erythrocytes on the 13th day. There was

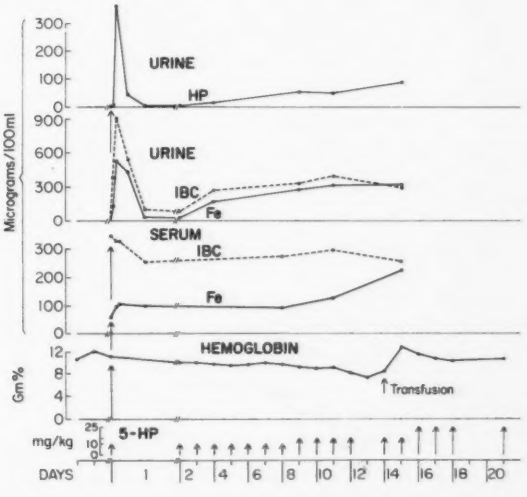

FIGURE 1.—Clinical observations in a 64-year-old man with carcinoma of the floor of the mouth who received 5-HP for 3 weeks.

no change in serum iron-binding capacity and an increase in serum iron; sampling was done in the early morning before each day's dose of 5-HP when the serum levels of 5-HP, though not measurable, were presumably very low.

Figure 2 represents the urinary excretion of iron during the continuous infusion of 5-HP, 25 mg daily for two periods of 5 and 3 days. There was clearly a marked increase in iron excretion beginning promptly at the start of the 5-HP infusion and returning to normal promptly at the end of the infusion. This patient is representative of 11 in whom serum iron and iron-binding capacity were followed serially during administration of 5-HP. In each, the serum iron level rose steadily and the iron-binding capacity remained stable. In each of these patients also, as in the patient represented in figure 1, the hemoglobin level fell steadily during 5-HP administration and multiple transfusions were administered. Although iron excretion was clearly excessive,

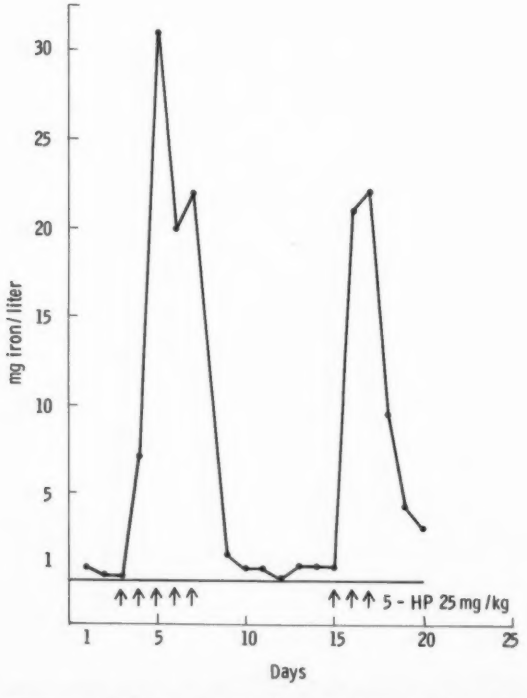

FIGURE 2.—Urinary excretion of iron during the continuous infusion of 5-HP.

the serum-iron levels rose. It must be concluded therefore that hemolysis is the most important facet of the effect of 5-HP on iron homeostasis and, though dramatic, the chelation and excretion of iron are of lesser significance than the hemolytic effect.

Figure 3 illustrates the influence of 5-HP on the excretion of iron 59 (^{59}Fe) in a 54-year-old woman with breast cancer who was given a dose of 50 microcuries of ^{59}Fe. The daily low level of urinary excretion of ^{59}Fe was observed. On the fifth day, a single dose of 5-HP (10 mg/kg) was given iv. There was a prompt and marked increase in the excretion of labeled iron reaching a peak of 58 cpm/ml and returning to low, nearly baseline levels within 24 hours. The administration of 5-HP in this study was followed, as usual, by the excretion of green urine due to the 5-HP:iron complex and by a fall in the hematocrit value from 31% to 28%, indicating some hemolysis. Coincident with the increase in iron excretion following 5-HP administration there was a slight but definite fall in the concentration of isotope in erythrocytes, confirming the evidence that the source of iron was largely from erythrocytes. It is not possible to be certain whether the release of iron (either labeled or unlabeled) following 5-HP administration is a consequence or a cause of hemolysis, since

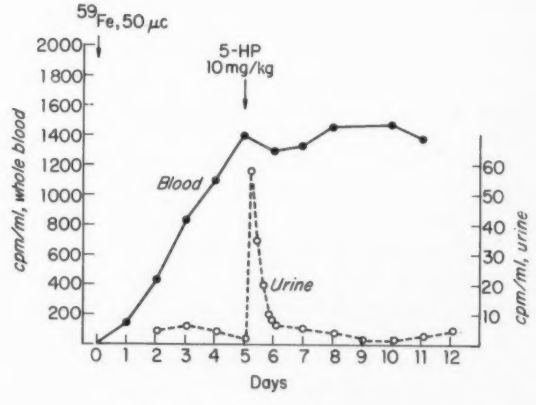

FIGURE 3.—Whole blood and urinary ^{59}Fe levels after a single dose of 50 microcuries of ^{59}Fe in a 54-year-old woman with breast cancer. A single dose of 5-HP (10 mg/kg) was given iv on the 5th day after the administration of ^{59}Fe.

the chelation of iron by 5-HP could occur after erythrocyte damage from another mechanism. Further studies to clarify this point will be undertaken.

Therapeutic Effects

An "adequate" course of 5-HP cannot be defined. Twenty patients who received at least 135 mg/kg manifested distinct hematologic abnormalities and, for the present, are considered to have received adequate trials. Three patients who had acute lymphoblastic leukemia achieved "good partial remissions," defined as a decrease in the percentage of blast cells in the bone marrow. However, other criteria of remission were not met, ie, improvement in hemoglobin level, platelet count, and peripheral differential white blood cell count. It is thought that these may not have been genuine remissions but that the erythroid hyperplasia secondary to the hemolytic effect resulted in a relatively temporary decrease in blast cells. There were no other therapeutic responses.

DISCUSSION

A disturbing aspect of the clinical use of 5-HP is the large number of side effects experienced by the patients. Although these are, for the most part, of short duration, they occur with unpredictable severity and do not necessarily parallel the dose.

The acute side effects closely mimic those observed with iron intoxication. It is therefore difficult to determine whether the effects are directly related to the drug or are a consequence of the mobilization of large amounts of iron which occurs regularly after 5-HP administration.

The level of urinary iron excretion after 5-HP administration is similar to that which might be achieved with other iron-binding compounds, such as deferoxamine (26).[7] However, 5-HP has been used in smaller doses than are customary for deferoxamine and it has not been used in patients with iron-storage disease. The severe nausea and vomiting associated with its use makes it unlikely that it could be usefully employed in the treatment of iron-storage diseases. It appears that 5-HP interferes with iron quantitation in the urine unless atomic absorption spectrometry is used.

The source of iron chelated by 5-HP cannot be determined from this study. The rapid development of a significant degree of anemia in some patients indicates that heme iron accounts for a substantial amount but non-heme stores may also contribute. It has not been determined whether 5-HP causes hemolysis and then binds the released iron or binds directly to heme iron, denaturing it and producing hemolysis. Presumably, its reactivity with iron accounts for the cytotoxic and antitumor effects in animals, since 5-HP, like hydroxyurea, is an inhibitor of ribonucleoside diphosphate reductase; it has been shown that this enzyme contains an iron moiety and the activity of hydroxyurea is specifically upon the iron-containing protein of the enzyme (14).

The responses to treatment were very poor. The three good partial remissions may not have been genuine partial remissions, since there was no improvement in platelet count or hemoglobin level. In fact, they may have been only the result of erythroid hyperplasia due to the hemolytic effect, with the appearance of a relative decrease in lymphoblasts. Similar feeble therapeutic activity and acute toxicity were observed by DeConti et al (16) in their phase I study.

The severe nausea and vomiting, the marked hemolytic effects at all dose levels, and the failure to demonstrate any significant therapeutic effect at any dose level do not suggest a useful role for 5-HP in cancer chemotherapy. However, analogs of this compound are being studied and it is possible that another thiosemicarbazone may prove to be a useful inhibitor of ribonucleoside diphosphate reductase without the limiting side effects characteristic of 5-HP.

[7] Deferoxamine: NSC-527604; CAS reg. No. 70-51-9; propionohydroxamic acid, *N*-[5-(3-[(5-aminopentyl)-hydroxycarbamoyl]propionamido) pentyl]-3-([5-(*N*-hydroxyacetamido)pentyl]carbamoyl)-.

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Vinblastine (NSC-49842) and Bleomycin (NSC-125066) Therapy for Disseminated Testicular Tumors [1,2]

Stuart C. Spigel and Charles A. Coltman, Jr.[3]

SUMMARY

A sampling of the accumulated experience of the treatment of disseminated nonseminomatous testicular carcinoma reveals an overall response rate of 35% with a complete response rate of only 10%. Vinblastine, a vinca alkaloid, and bleomycin, a new antitumor antibiotic, have both demonstrated significant activity against this disease. A pilot study was initiated at Wilford Hall United States Air Force Medical Center to test the efficacy of these two drugs used in combination. Eleven patients were treated, resulting in five complete and four partial responders, with two patients showing no response. All complete responders have been maintained on vinblastine alone and no relapses have occurred in this group within a maximum followup duration of 13+ months. In general, toxicity has been mild, with no pulmonary manifestations. The postulated reason for the efficacy of this drug combination is discussed.

[Cancer Chemother Rep 58:213–216, 1974]

Although testicular tumors comprise only 1.5%–2% of all malignant tumors in men, 80% of these neoplasms occur between the ages of 18 and 44 and constitute 13% of all cancers in men between the ages of 20 and 40. Although pure seminomas are very sensitive to both radiotherapy and chemotherapy and 5-year survival is over 90%, the embryonal cell carcinomas, teratomas, choriocarcinomas, and mixtures of these cell types are very resistant tumors. In a review of 555 patients with metastatic nonseminomatous testicular cancer treated according to eight different protocols (1–4) that utilized drugs both singly and in combination, the overall response rate is only 35% with a complete response rate of 10%.

Vinblastine, a plant alkaloid, and bleomycin, a soil fungus antitumor antibiotic, have individually shown efficacy in testicular cancer. Samuels (5) has documented seven partial and four complete responses in 21 patients treated with vinblastine. Bleomycin has been associated with 11 partial responses and one complete response in 37 treated patients (6). It was reasonable then, to combine these agents in full doses because their spectra of toxicities do not overlap. Samuels conducted the initial such trial of this drug combination and the results suggested true therapeutic synergism (6). Seventeen of 19 patients responded with three patients having complete responses. In a subgroup of ten embryonal cell patients treated with both drugs there were ten responses, while in a group of 21 embryonal cell patients treated with bleomycin alone there were only eight responses. On the basis of these data, a

[1] Received Mar 30, 1973; revised Aug 20, 1973; accepted Sept 19, 1973.

[2] Vinblastine: CAS reg. No. 6449-03-2; vincaleukoblastine, sulfate (1:1), monohydrate. Vinblastine and bleomycin were supplied by Cancer Therapy Evaluation, Division of Cancer Treatment, National Cancer Institute.

[3] Hematology-Oncology Service, Wilford Hall USAF Medical Center, Lackland Air Force Base, San Antonio, Tex.

pilot study utilizing these agents was instituted at Wilford Hall United States Air Force Medical Center in the summer of 1971. Eleven patients have since been accumulated and form the substance of this report.

METHODS

All patients with metastatic testicular cancer were eligible regardless of prior radiotherapy or chemotherapy, except for those who had had a trial with one of the protocol drugs. Patients must have had clinically or radiographically discrete measurable lesions, estimated survival rates of at least 7 weeks, and near normal hepatic and renal functions.

During the study period 13 patients with metastatic testicular cancer were seen in the Oncology Service. Eleven were eligible for the vinblastine-bleomycin protocol. One was excluded because of his terminal status and another was excluded because of halothane hepatitis. Table 1 reflects the composition of the study group. Figure 1 outlines the treatment plan. A dose ceiling of 400 mg was observed for bleomycin, and for patients receiving extensive prior radiotherapy vinblastine was initiated at half dose to avert unacceptable myelosuppression. Vinblastine doses were escalated by increments according to the white blood cell and platelet counts. Patients with increasing disease at any point were dropped from the study. Response criteria are summarized in figure 2.

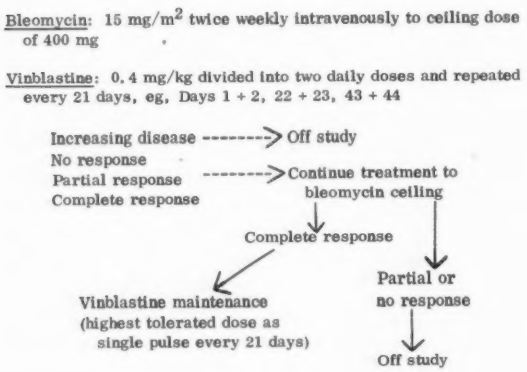
Bleomycin: 15 mg/m² twice weekly intravenously to ceiling dose of 400 mg
Vinblastine: 0.4 mg/kg divided into two daily doses and repeated every 21 days, eg, Days 1 + 2, 22 + 23, 43 + 44
Increasing disease
Off study
No response
Partial response
Complete response
Continue treatment to bleomycin ceiling
Complete response
Vinblastine maintenance (highest tolerated dose as single pulse every 21 days)
Partial or no response
Off study

FIGURE 1.—Chemotherapeutic regimen for bleomycin and vinblastine.

TABLE 1.—Characteristics of the study group

	No. of patients
Histologic type	
Embryonal	5
Teratocarcinoma	2
Embryonal + choriocarcinoma	2
Teratocarcinoma + embryonal + choriocarcinoma	2
Locus of metastatic disease	
Lung alone	6
Lung + nodes	3
Abdominal mass in radiated field	2
Prior therapy	
None	4
Retroperitoneal node dissection + cobalt 60	3
Retroperitoneal node dissection	1
Cobalt 60 + chemotherapy	1
Chemotherapy	1
Chemotherapy + retroperitoneal node dissection + cobalt 60	1
Total No. of patients treated (median age: 28 years)	11

RESULTS

Five complete and four partial responses were elicited for an overall response rate of 88%. Two patients demonstrated no response; they had previously received extensive radiotherapy and were unable to tolerate more than half doses of vinblastine due to myelosuppression. Three partial responders had prior chemotherapy. On the other hand no complete responder had previously received chemotherapy and only one had received radiotherapy. Response data are summarized in table 2.

All complete responders had apparent metastatic disease limited to the lung except one patient who also had left supraclavicular node involvement. Three of the four partial

Complete response: Disappearance of all measurable or apparent tumor and normalization of chorionic gonadotropin titers

Partial response: > 50% decrease in the sum of the products of the diameters of measured lesions

No response: < 50% decrease in the sum of the products of diameters of measured lesions or no change in size of measurable or otherwise apparent tumor

Increasing disease: > 50% increase in the sum of the products of the diameters of measured lesions or appearance of new tumor

FIGURE 2.—Summary of response criteria.

TABLE 2.—Summary of experience with vinblastine and bleomycin

Patient No.	Cell type*	Prior therapy			Response			Duration of response (mos)
		RND†	Cobalt 60	Chemotherapy	Complete	Partial	None	
1	E	×	0	0	×			13+
2	T,E,C	0	0	0	×			6+
3	E	0	0	0	×			6+
4	E,C	×	×	0	×			2+ (LFU)‡
5	E	0	0	0	×			2+ (LFU)
6	E	0	×	×		×		3
7	E,C	×	×	×		×		5
8	T	0	0	0		×		3
9	T	0	0	×		×		Early death, cause unknown
10	E	×	×	0			×	—
11	T,E,C	×	×	0			×	—

*E = embryonal cell; T = teratocarcinoma; C = choriocarcinoma.
†RND = retroperitoneal node dissection.
‡LFU = lost to followup.

responders had extrapulmonary disease; two of these had retroperitoneal masses.

All complete responses were apparent by 4 weeks and the median time for complete response was 6 weeks. No complete responder has relapsed with a maximum duration of complete response of 13+ months. All complete responders have well tolerated outpatient maintenance with vinblastine every 3 weeks.

Toxic effects were generally mild and tolerable consisting largely of skin blistering, fingertip swelling and dysesthesia, and stomatitis, all of which were attributable to bleomycin. No pulmonary toxicity of the type associated with bleomycin was noted although one patient (No. 7) received 1300 mg in support of a prolonged (>90%) response. The only severe toxic effects that occurred were observed in four of the five patients who had extensive prior radiotherapy. These patients experienced dose-limiting leukopenia attributable to vinblastine, and two patients had brief granulocytopenic episodes of less than 1000 cells/mm^3 terminating in gram-negative sepsis. No deaths were charged to toxicity.

DISCUSSION

The results of this pilot study support the hypothesis that vinblastine and bleomycin yield superior results in the induction of remission in disseminated nonseminomatous testicular cancer. Five of 11 patients treated demonstrated complete response, a rate of 45% as compared to the 10% figure culled from the overall literature reviewed. Though the number of cases is small, our data support those of Samuels, and it is unlikely that the combined results of both studies are due to chance. An additional finding is the efficacy of vinblastine in maintaining complete responses. Samuels data (5) reveal that complete responders who receive no maintenance therapy relapse in a mean of 2.1 months. The vinblastine-maintained complete responders in this series have not relapsed and have had a maximum duration of response of 13+ months.

Unlike the experience with mithramycin,[4] a drug whose efficacy is virtually limited to embryonal cell carcinoma, responses in the

[4] Mithramycin: NSC-24559; CAS reg. No. 18378-89-7.

current study occurred in all tumor types exposed. Indeed, the factor which seemed to be most important in predicting response was lack of prior therapy, a phenomenon noted in other classes of neoplasm, notably Hodgkin's disease (7).

The reason for the apparent efficacy of the vinblastine-bleomycin combination is not entirely clear. Barranco and Humphrey (8) have presented evidence that bleomycin is most effective at killing cells in mitosis and vinblastine is a potent mitotic arresting agent. In addition then to the cells that each drug destroys individually, their combination may kill an additional increment of cells only marginally damaged by vinblastine but still viable and clonogenic. This implies that the combination is synergistic, an hypothesis hinted at but certainly not proved by existing data.

Umezawa et al (9) have shown a preferential accumulation of bleomycin in lung and skin, an observation which may relate to the seemingly better aggregate response in the group with disease in lung alone.

The results in this small group of patients support the need for a comparative study of this versus other accepted active treatment programs for a large number of patients.

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Clinical Trial With Subcutaneously Administered 5-Azacytidine (NSC-102816) [1,2,3]

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SUMMARY

5-Azacytidine was administered subcutaneously (sc) to 22 patients with a variety of advanced solid malignant tumors for 10 successive days to a total dose of 275–850 mg/m². Objective tumor regressions were seen in two patients: one with adenocarcinoma of the breast and one with adenocarcinoma of the ovary. Mild gastrointestinal toxicity was noted at doses producing significant bone marrow depression. Of particular interest was the observation of hepatic toxicity in five patients, three of whom went into hepatic coma. Sc administration of 5-azacytidine is a useful alternative to the standard intravenous route of administration, especially in patients with inadequate sites for venipuncture.

[Cancer Chemother Rep 58:217–222, 1974]

[1] Received June 20, 1973; accepted Sept 21, 1973.

[2] Supported by Public Health Service (PHS) grants CA-12291, CA-06071, CA-06551, and CA-05255 (National Cancer Institute [NCI]) and RR-05539 (Division of Research Resources), National Institutes of Health (NIH), Department of Health, Education, and Welfare (DHEW); by contract NIH-70-2136 from the Division of Cancer Treatment (DCT), NCI, NIH, PHS, DHEW; and by an appropriation from the Commonwealth of Pennsylvania.

[3] 5-Azacytidine was supplied by Cancer Therapy Evaluation, DCT, NCI. Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are listed in the List of Compounds at the end of the text.

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[9] We wish to thank Dr. Richard Bornstein for entering patients into this clinical trial.

5-Azacytidine is the pyrimidine analog of cytidine with a nitrogen atom substituted for carbon in the 5-position of the ring (fig 1). Píškala and Šorm (1) synthesized this nucleoside in 1964; 2 years later, Hanka et al (2) isolated it from a culture of *Streptoverticillium ladakanus*. 5-Azacytidine has been studied in many in vitro systems and potent antibacterial (3–5), mutagenic (6, 7), and cytostatic (8) activity has been demonstrated. Mechanisms of action of 5-azacytidine include competition for uridine kinase (9), inhibition of orotidylic acid decarboxylase (10), incorporation into RNA (11,12), and incorporation into DNA (12,13). This antimetabolite has shown significant antitumor activity against murine L1210 leukemia, Walker 256 carcinosarcoma,[10] and acute lymphoblastic leukemia in AK mice (14). On the basis of these preclinical studies, 5-azacytidine was chosen for trials in man.

A phase I study of intravenously (iv) administered 5-azacytidine using a total dose of

[10] Broder LE, and Carter SK. Clinical brochure: 5-azacytidine (NSC-102816), Oct 1970, 25 pp. Prepared by the NCI, Bethesda, Md.

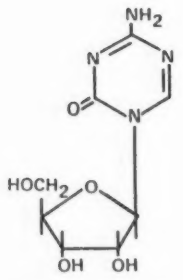

FIGURE 1.—Structure of 5-azacytidine.

300–700 mg/m² revealed that the drug has gastrointestinal and hematologic toxic effects (15). The severe incapacitating emesis resulting from the iv administration of 5-azacytidine and the lack of local tissue reaction upon extravasation provided the impetus for this investigation of an alternate route of administration. Furthermore, in a comparison of iv and subcutaneous (sc) administration of 5-azacytidine (16), plasma levels were not significantly different 1 hour after injection and preferential concentration in tumor tissue was demonstrated regardless of the route of administration.

MATERIALS AND METHODS

5-Azacytidine was administered to 22 patients with a variety of advanced malignant tumors. Histologic evidence of malignancy and a measurable lesion were prerequisites for entry into this study. Patients who were expected to live less than 3 months and patients who had had previous total pelvic irradiation, prior adrenalectomy and/or hypophysectomy, or irradiation or chemotherapy within 30 days of the start of the study were excluded from the study. Also excluded were patients who had a hemoglobin value less than 9 g/100 ml, a white blood cell count (wbc) less than 4000 cells/mm³, a platelet count less than 100,000 platelets/mm³, a blood urea nitrogen (BUN) value greater than 25 mg/100 ml, a bilirubin value greater than 1.5 mg/100 ml, or a serum glutamic oxaloacetic transaminase (SGOT) value greater than 120 International Units (IU) (normal value, 5–40 IU).

Eight men and 14 women were included in this study. The mean age of the men was 63 years (range, 49–77 years) and the mean age of the women was 58 years (range, 33–74 years) (table 1). Patients were treated with 5-azacytidine at a dose of 275–850 mg/m² given sc for 10 successive days as initial therapy. Patients who responded to initial therapy received maintenance treatment with 5-azacytidine at a dose of 35–90 mg/m²/week.

Palpable lesions were measured weekly. Pulmonary parenchymal masses were evaluated with weekly chest X-ray films and hepatic lesions were evaluated with biweekly liver scans.

Hemoglobin determinations, wbc counts, and platelet counts were performed on Days 1, 5, and 10 of therapy and thereafter at weekly intervals. BUN, bilirubin, and SGOT values were determined weekly.

5-Azacytidine was supplied in sterile vials containing 50 mg of white lyophilized powder. The contents of each vial were reconstituted with 3 ml of sterile water immediately prior to injection.

RESULTS

Toxicity

Four patients died of progressive disease prior to the completion of the 6-week followup period and are excluded from the toxicity evaluation. The toxicity experienced by the 18 evaluable patients is summarized in table 2. In six patients there were no toxic effects; in 12 patients definite single or multiple toxic effects occurred. One patient had severe stomatitis. No dermatitis, alopecia, or renal impairment was noted.

Twelve patients experienced anorexia during the 10-day loading regimen; eight of these patients had nausea, which was generally well tolerated. Only four patients experienced drug-related vomiting. Vomiting began 2–3 hours after injection, was controlled by moderate doses of antiemetics, and cleared in 3–4 hours. Tolerance to nausea and vomiting developed in all patients as additional doses of 5-azacytidine were administered so that by Day 10 of the loading regimen no vomiting was noted. Three patients had drug-related episodes of diarrhea which were self-limited and mild.

Deterioration in liver function was seen in

TABLE 1.—Summary of data for evaluable patients treated with 5-azacytidine

Patient No.	Age,sex	Tumor	Prior therapy*	Total dose of 5-azacytidine	
				Mg/m²	Mg
1	71,F	Ovary	S, cyclophosphamide, adriamycin	275	400
2	62,F	Ovary	S	345	500
3	61,F	Ovary	S, R, cyclophosphamide	370	500
4	64,F	Breast	S, R, cyclophosphamide	385	500
5	63,F	Tonsil	S, R	417	500
6	56,M	Lung	S, cyclophosphamide, methotrexate	333	525
7	74,F	Breast	S, R	375	600
8	49,M	Hepatoma	S, 5-FU	441	750
9	51,F	Ovary	S	500	750
10	67,M	Colon	S, R, 5-FU	513	1000
11	58,M	Hypernephroma	S, hydroxyurea, adriamycin	587	1000
12	63,M	Melanoma	S, NSC-45388	588	1000
13	67,F	Breast	S	667	1000
14	49,F	Breast	S	689	1000
15	59,F	Colon	S, 5-FU	806	1250
16	64,M	Colon	S	769	1500
17	77,M	Colon	S	850	1500
18	58,M	Chordoma	S, adriamycin	850	1500

*S = surgical treatment; R = radiation therapy.

TABLE 2.—Toxicity of 5-azacytidine in 18 evaluable patients*

Toxic effect	No. of patients
No toxicity	6
Toxicity	12
Gastrointestinal (total of 12 patients)	
Anorexia	12
Nausea	8
Vomiting	4
Diarrhea	3
Hepatic (total of 5 patients)	
Liver function abnormalities	5
Hepatic coma	3
Hematologic (total of 12 patients)	
Leukopenia	12
Thrombocytopenia	3
Anemia	2
Stomatitis	1
Deaths	5

*Of 22 patients treated with 5-azacytidine, 18 were evaluable for toxicity.

five patients. This consisted of elevations of total bilirubin and/or SGOT levels to at least three times the pretreatment values (baseline bilirubin value, <1.5 mg/100 ml; baseline SGOT value, <120 IU). The three patients who had both abnormal total bilirubin values and abnormal SGOT values died in hepatic coma.

Hepatic coma was diagnosed when a patient manifested lethargy, confusion, icterus, and asterixis with elevated values for bilirubin and SGOT but with normal values for BUN, blood glucose, serum electrolyte concentrations, and serum calcium. All patients in whom hepatic coma developed experienced a rapidly progressing downhill course and died within 10 days of the onset of icterus. Significant hepatic tumor burden had been confirmed in these patients by liver scan and needle biopsy prior to therapy. Hepatic toxicity did not appear to be dose related.

TABLE 3.—Summary of the cases of patients who died of 5-azacytidine toxicity

Patient No.	Age,sex	Tumor	Total dose (mg/m^2)	Cause of death
1	71,F	Adenocarcinoma, ovary	276	Gastrointestinal bleeding secondary to thrombocytopenia
3	61,F	Adenocarcinoma, ovary	370	Hepatic coma
4	64,F	Adenocarcinoma, breast	385	Central nervous system bleeding secondary to thrombocytopenia
10	67,M	Adenocarcinoma, colon	513	Hepatic coma
12	63,M	Melanoma	588	Hepatic coma

Twelve patients experienced a dose-related fall in wbc to less than 50% of pretreatment values. The nadir of the wbc occurred on Day 27 (mean) (range, 21–35 days). Wbc counts returned to normal 10 days (mean) thereafter. Four patients experienced significant leukopenia (wbc <2000 cells/mm^3); however, none of the patients had fever or signs of systemic infection. Three patients had platelet counts less than 50,000 platelets/mm^3 with petechiae. All three patients subsequently died of drug toxicity. Two deaths were directly attributable to thrombocytopenia. Severe anemia (hemoglobin level <5 g/100 ml) occurred in two patients because of marrow hypoplasia. Both patients were pancytopenic and both died (one of thrombocytopenia and the other of hepatic coma).

Five patients died of drug toxicity (table 3). All five patients were more than 60 years of age and had received prior chemotherapy; the 5-azacytidine dosage was moderate, ie, 276–588 mg/m^2 total dose.

Response to Therapy

Only two of the 18 evaluable patients experienced clinically useful partial remissions (defined as a decrease of more than 50% in the volume of measurable lesions for 1 month). Both responding patients (one with adenocarcinoma of the breast and one with adenocarcinoma of the ovary) received maintenance therapy with weekly doses of 5-azacytidine and remained in remission for 2 and 3 months, respectively. Remissions, when they occurred, were early and dramatic (fig 2).

DISCUSSION

In this study of sc administered 5-azacytidine, leukopenia and thrombocytopenia were dose related and delayed (21–35 days after the start of treatment). In spite of severe leukopenia, infection was not evident in any patient. However, the appearance of thrombocytopenia signaled a serious hemorrhagic diathesis.

Liver function abnormalities were demonstrated in five of our 18 evaluable patients; three of these patients (all with significant hepatic metastatic disease) died of rapidly progressing hepatic coma. In view of the focal hemorrhagic necrosis and fatty metamorphosis reported to occur in the livers of dogs treated with toxic doses of 5-azacytidine,[11] a prospective study to evaluate the effects of this agent on the human liver was undertaken (17). Histologic evaluation of coded percutaneous needle biopsy specimens of liver from eight patients in whom there was no evidence of hepatic metastatic disease revealed no significant differences between pretreatment and posttreatment specimens. Nevertheless, the recent revelation of preferential and prolonged concentration of this potentially hepatotoxic drug in the liver (Troetel, W. M., personal communication) makes it clear that caution should be exercised in treating patients with significant hepatic tumor burden. Specifically, 5-azacytidine therapy is probably contraindicated in patients in whom replacement of liver tissue by tumor is extensive and who

[11] *See* footnote 10.

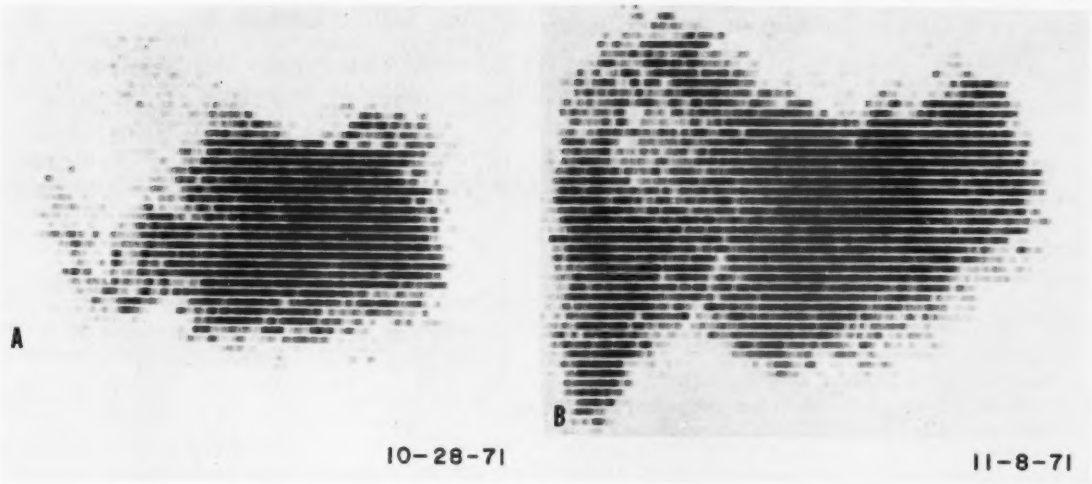

FIGURE 2.—^{99m} Technetium sulfur colloid liver scans of patient No. 2 who had adenocarcinoma of the ovary metastatic to the liver. *A*. Pretreatment scan reveals large filling defects in the superior and lateral inferior aspects of the right lobe of the liver. *B*. Posttreatment scan demonstrates significant improvement in isotope uptake consistent with tumor regression.

have elevated bilirubin values, an SGOT value greater than 120 IU, or a serum albumin value less than 3.0 g/100 ml (17).

Liver function abnormalities and hematologic toxicity are consistent with the findings of other investigators who have administered therapeutic courses of 5-azacytidine (15,18,19). The high incidence of hepatic coma in the present study probably reflects the selection of patients with significant hepatic metastatic disease.

A number of investigators (15,18–21) have been dismayed by the severe dose-limiting gastrointestinal toxicity of 5-azacytidine given iv. Moertel (18, p 652) has stated that "the severity of nausea and vomiting induced by 5-azacytidine seriously compromises any hope of clinical usefulness." However, the sc route produced only mild gastrointestinal toxic effects at doses sufficient to cause significant bone marrow depression.

With respect to antitumor activity, only two patients (one with adenocarcinoma of the breast and one with adenocarcinoma of the ovary) had clinically useful partial remissions. Limited therapeutic activity in solid tumors is consistent with three recent studies reporting objective remissions in two of 21 patients with carcinoma of the breast (20), none of four patients with melanoma (19), and one of 27 patients with cancer of the colon (18). The response rates reported by Weiss (15) (seven of 11 patients with carcinoma of the breast, two of five patients with melanoma, and two of six patients with cancer of the colon) probably reflect the high percentage of patients with soft tissue metastatic disease entered into the study.

Karon et al (21) reported significant antitumor activity of 5-azacytidine in patients with acute childhood leukemia resistant to conventional therapy. Our demonstration of mild gastrointestinal toxicity with significant bone marrow suppression after sc administration provides a useful alternate route of administration of 5-azacytidine to children in whom appropriate sites for venipuncture may be lacking.

CONCLUSIONS

The daily sc administration of 5-azacytidine for 10 days to a total dose of 275–850 mg/m^2 resulted in mild gastrointestinal toxic-

ity and dose-limiting leukopenia. The development of fatal hepatic coma in three patients indicates that care should be exercised in treating patients who have significant hepatic tumor burden. Clinically useful tumor regressions were noted in one of four patients with cancer of the breast, one of four patients with cancer of the ovary, and none of four patients with cancer of the colon. The sc route of administration of 5-azacytidine is a useful alternative to the iv treatment route.

LIST OF COMPOUNDS

Adriamycin: NSC-123127

5-Azacytidine: NSC-102816; CAS reg. No. 320-67-2; *s*-triazin-2(1*H*)-one, 4-amino-1-β-D-ribofuranosyl-

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate

5-FU: NSC-19893; CAS reg. No. 51-21-8; 5-fluorouracil

Hydroxyurea: NSC-32065; CAS reg. No. 127-07-1

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]-methylamino]benzoyl]-

NSC-45388: CAS reg. No. 4342-03-4; imidazole-4-carboxamide, 5-(3,3-dimethyl-1-triazeno)-; DIC

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Dibromomannitol (NSC-94100): A Clinical Study of Previously Treated Patients With Refractory Chronic Myelocytic Leukemia and Blastic Transformation [1,2,3]

William C. Levin,[4] Charles H. Mims,[4] and Arthur Haut [5]

SUMMARY

The effectiveness of dibromomannitol in the treatment of blastic transformation and refractory chronic myelocytic leukemia (CML) was evaluated. Fourteen patients with blastic transformation and 12 patients with CML resistant to busulfan were studied. Therapy consisted of treatment with dibromomannitol at a dose of 250 mg/m² for 3 days and, thereafter, 125 mg/m² daily. Peripheral blood cell counts and bone marrow morphology were monitored. Although dibromomannitol appeared to be effective in decreasing the peripheral white blood cell count, no objective remissions were observed. The main toxic effects were severe leukopenia and thrombocytopenia, both of which were reversible. Dibromomannitol does not appear to be therapeutically effective in patients with blastic transformation or busulfan-resistant CML.

[Cancer Chemother Rep 58:223–228, 1974]

Dibromomannitol, a halogenated sugar alcohol, was first reported to have cytotoxic activity in 1961 (1). Since then, there have been several reports attesting to its effectiveness in the treatment of myeloproliferative disorders, especially chronic myelocytic leukemia (CML) (2–9).

In 1969, the Southwest Cancer Chemotherapy Study Group began a study to test the activity of dibromomannitol in the management of blastic transformation of CML and in the management of CML that had become refractory to busulfan.

DRUG INFORMATION

The chemical structures of busulfan, mannitol myleran, and dibromomannitol are shown in figure 1. In an attempt to discover another agent effective against neoplastic diseases, the chemical structure of busulfan was changed. Several polyalcohol compounds in which the methanesulfonyl group of busulfan was replaced were studied. Dibromomannitol was synthesized by substituting bromine, which may behave like the methanesulfonyl group. The mechanism of action of dibromomannitol is uncertain. In vitro, it behaves like an alkylating agent (1), but in vivo studies fail to document this (10). It has been concluded that

[1] Received July 20, 1973; accepted Sept 25, 1973.

[2] This study was performed by the Southwest Cancer Chemotherapy Study Group and was supported by Public Health Service grants CA-03096 (William C. Levin), CA-03400 (Arthur Haut), CA-04919 (James S. Hewlett), CA-04920 (Henry E. Wilson), CA-03389 (W. J. Stuckey), CA-13238 (Joseph M. Quagliano), CA-10187 (John D. Bonnet), and CA-04915 (Raymond W. Monto) from the National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3] Dibromomannitol was supplied by Cancer Therapy Evaluation, Division of Cancer Treatment, NCI. Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are listed in the List of Compounds at the end of the text.

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dibromomannitol has the properties of both an alkylating agent and an antimetabolite.

Thirty minutes after dibromomannitol is ingested, 95% of the drug is absorbed and present in the portal venous system (10). A portion of the drug is present in the systemic circulation, but a significant quantity enters the enterohepatic circulation. This latter pathway allows drug levels in the blood and tissues to remain elevated for approximately 24 hours after a single dose (10).

After dibromomannitol had been synthesized, it was initially studied in various animal tumor systems and an almost selective depression of the granulocytic elements was observed. Hence, within approximately 1 year of its synthesis and study in animals, dibromomannitol was used in the treatment of patients with CML (3). In the initial studies, approximately 70%–80% of the patients with CML had a partial or complete remission (3–5). Both intermittent (4,6) and continuous (2,3,7–9) therapeutic regimens have been used, and present evidence indicates that these approaches are equally effective and equally toxic. In the intermittent regimens, dibromomannitol is given at a dose of 4–6 mg/kg for 3–7 days as often as is necessary to keep the white blood cell count (wbc) at normal levels. The continuous regimens usually consist of an initial dose of 250–500 mg daily until there is a significant reduction in the total leukocyte count; this reduction usually occurs within 2–12 weeks after the start of therapy. Maintenance treatment usually consists of 250-mg doses given two to three times weekly.

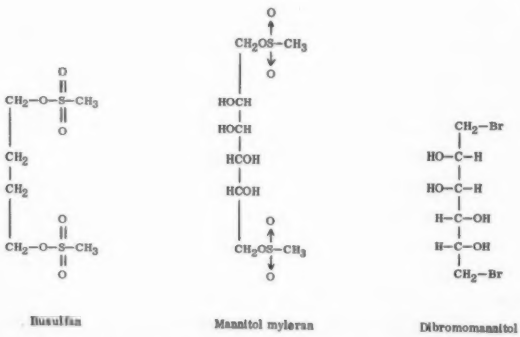

FIGURE 1.—Structures of busulfan, mannitol myleran, and dibromomannitol.

Only two reports have commented in any detail on the length of remission (3,6). In patients treated with a continuous regimen at a dose somewhat higher than that stated previously, the duration of remission ranged from 1 to 24 months (3). When an intermittent regimen was used, similar results were obtained (6). The number of cases reported and the length of followup are not such that they permit an estimation of the effect of dibromomannitol on the prolongation of life.

Since the initial clinical trials with dibromomannitol, investigators have emphasized its "selective" granulocytotoxic activity (3,5). In the initial clinical trial, dibromomannitol caused a reduction in erythrocytes and thrombocytes in 13% and 8% of the cases, respectively (3). The anemia and thrombocytopenia were said to be of short duration and they were not considered to be clinically significant. Other investigators have commented on the infrequency of this side effect, and it is their opinion that dibromomannitol is particularly indicated in those cases of CML that are complicated by anemia or thrombocytopenia or both (6). However, in the experience of Ramanan and Israëls (8), thrombocytopenia, particularly in those patients who had received other therapy previously, was a factor limiting the use of dibromomannitol. Tura and Baccarani (9) described a more favorable experience regarding the frequency and severity of thrombocytopenia. Leukopenia appears to be dose dependent. It has been recommended that the dose of dibromomannitol be reduced or discontinued when the leukocyte count falls to less than 20,000 cells/mm^3 since the fall continues for several days after the cessation of administration of the drug. Leukopenia has persisted for as long as 8 weeks after the cessation of drug therapy (2). Dibromomannitol has also been reported to be effective in reducing splenomegaly (2–4).

All of these studies indicate that response to dibromomannitol treatment is not influenced by patient age, duration of disease, or previous therapy. Busulfan has been reported to be ineffective in patients treated initially with dibromomannitol (11).

Petrányi and Bobory (6) expressed the opinion that the acute terminal phase of a

blastic transformation may be modified by dibromomannitol, but Tura and Baccarani (9) reported that, as is the case with busulfan, resistance to dibromomannitol occurs in blastic crisis.

One study comparing busulfan and dibromomannitol has been reported (11). This study emphasized mainly toxicity and mechanism of action. The question of therapeutic superiority of one agent over the other has not been adequately tested. Nonhematologic toxicity attendant upon dibromomannitol administration appears to be relatively infrequent when small doses are used (2). Investigators who have used larger doses have reported the occurrence of alopecia, nausea, vomiting, anorexia, diarrhea, abdominal pain, and pruritus (5,6).

MATERIALS AND METHODS

Patients eligible for this study were divided into two groups as follows:

1. The *refractory group* included (*a*) patients with previously diagnosed CML who had been treated with busulfan at a dose of at least 8 mg/day and whose leukocyte counts had failed to fall to less than 50% of the initial level within 4 weeks, or (*b*) patients who had previously had a remission and whose diseases had become refractory to busulfan (as reflected by an increase in the level of leukocytes from 25,000 cells/mm^3 or more) during treatment with the drug.
2. The *myeloblastic transformation group* included patients who had previously satisfied the diagnostic criteria for CML and who had been adequately treated. They were eligible for admission to the study after a relapse had occurred in association with 30% or more myeloblasts and promyelocytes in the peripheral blood or 50% or more myeloblasts and promyelocytes in the bone marrow.

The starting dose was dependent upon the initial wbc. In patients with a wbc of more than 50,000 cells/mm^3, treatment was initiated at a dose of 250 mg/m^2 daily for 3 days, followed by daily treatment with 125 mg/m^2 until the wbc fell to less than 10,000 cells/mm^3. In patients with leukocyte counts less than 50,000 cells/mm^3, the dosage was 125 mg/m^2/day. This was continued until the leukocyte count fell to less than 10,000 cells/mm^3. When the leukocyte counts fell to less than 10,000 cells/mm^3, treatment was continued at a dose level sufficient to keep the wbc between 5000 and 10,000 cells/mm^3. The dosage was reduced by one half if the leukocyte count was between 4000 and 5000 cells/mm^3. If the wbc was less than 4000 cells/mm^3, treatment was discontinued. If no biologic effect was observed after 3 weeks, the dosage was increased to 200 mg/m^2/day in both groups. If no biologic effect occurred within another 3 weeks, treatment was discontinued. Since the platelet count is often very low during blastic transformation, treatment was started without consideration of this parameter.

All patients were subjected to a detailed prestudy evaluation which included history and physical examination and the following laboratory examinations: (*a*) peripheral blood cell counts; (*b*) bone marrow examinations; (*c*) karyogram for the presence of Philadelphia (Ph^1) chromosomes; and (*d*) determination of values for leukocyte alkaline phosphatase, blood urea nitrogen, serum uric acid, serum bilirubin, serum alkaline phosphatase, and serum glutamic oxaloacetic transaminase.

RESULTS

Fourteen patients with blastic transformations were admitted to this study. The data are summarized in table 1. In each instance, previous treatment is indicated, and the total dose of dibromomannitol administered (in milligrams) is noted. Maximum reductions in wbc, percent of blasts, and changes in the size of the spleen and liver are recorded. Although there were impressive reductions in total leukocyte counts and, sometimes, in the percentage of blasts in the peripheral blood, there was no clinical evidence of improvement in any of these patients. No remissions were induced and in no instance was there demonstrable extension of life.

Twelve patients with busulfan-resistant CML were admitted to the study. The data obtained for these patients are summarized in table 2. Significant reductions in leukocyte counts oc-

TABLE 1.—Response to treatment with dibromomannitol in patients with myeloblastic transformation

Patient No.	Age, sex	Previous treatment	Total dibromomannitol dose (mg)	Wbc (cells × 10^3)		Blasts (%)		Spleen (cm below left costal margin)		Liver (cm below right costal margin)	
				Initial	Lowest	Initial	Lowest	Before treatment	Smallest during treatment	Before treatment	Smallest during treatment
1	55,M	Busulfan	2000	48	19	45	20	24	16	—	—
2	61,F	Busulfan	6400	190	28	15	12	28	4	0	1
3	62,M	Busulfan	8900	123	9	19	17	2	2	2	2
4	11,M	Busulfan	4600	39.5	0.385	32	0	0	0	0	0
5	45,F	Busulfan, prednisone	3075	94.1	11.6	39	28	10	10	8	9
6	51,M	Busulfan	1500	44.3	0.3	31	7	10	6	0	0
7	19,M	Busulfan	5225	74.4	0.3	64	48	—	—	4	—
8*	71,M	None	8775	42.4	0.7	85	27	2	0	0	0
9	20,M	Busulfan	9600	20.7	3.2	—	2	0	0	0	0
10	60,M	Busulfan	2500	29	34	53	66	—	—	—	—
11	33,M	Busulfan	1750	76	1.6	70	8	8	—	—	—
12†	18,M	Busulfan	17,750	247	32.9	3	8	3	0	3	0
13	33,M	Busulfan	3300	173	3.1	0	3	18	14	7	—
14‡	53,M	None	6250	43	9.1	43	61	14	13	—	—

*Myelofibrosis diagnosed in 1969.
†Bone marrow 65% blasts and promyelocytes.
‡Basophilia (14%) and Philadelphia chromosome positive.

TABLE 2.—Response to treatment with dibromomannitol in patients resistant to busulfan

Patient No.	Age, sex	Previous treatment	Total dibromomannitol dose (mg)	Wbc (cells × 10^3)		Blasts (%)		Spleen (cm below left costal margin)		Liver (cm below right costal margin)	
				Initial	Lowest	Initial	Lowest	Before treatment	Smallest during treatment	Before treatment	Smallest during treatment
1	59,F	Radioactive phosphorus (^{32}P), azathioprine, busulfan	13,585	123	34.5	0	1	0	0	0	0
2	43,M	None	2350	143	305	—	—	15	15	0	0
3	78,M	Busulfan	1600	52.5	27.5	0	0	5	—	0	—
4	31,M	Busulfan	27,490	114	4.3	—	—	12	11	—	—
5	62,M	Busulfan, 6-mercaptopurine	52,050	284	30.1	—	—	18	11	—	—
6	63,F	Busulfan	6650	43.3	11.8	7	4	8	—	15	—
7	51,F	Busulfan	10,100	224	2.7	2	0	26	20	16	12
8	45,F	Busulfan	6600	250	65.9	0	1	3	6	4	2
9	50,M	Busulfan, prednisone, vincristine	9225	109	10.2	0	—	10	10	4	4
10	59,M	Busulfan	17,475	13	2.3	0	0	0	0	0	0
11	81,F	Melphalan	1900	151	5.9	6	0	—	—	—	—
12	70,M	Busulfan, 6-mercaptopurine	4950	146	66.8	8	2	3	3	—	—

curred in seven of the 12 patients. Among these seven patients, reduction in spleen size was quite inconstant and there was no clinical evidence of a remission induction or extension of survival.

Toxicity was manifested chiefly by the development of leukopenia and thrombocytopenia. Significant thrombocytopenia developed in 11 of the 26 patients with platelet counts as low as 300 platelets/mm^3 occurring. Despite the discontinuance of treatment when leukocyte counts fell to less than 4000 cells/mm^3, eight of the 11 patients had leukopenia with leukocyte counts varying from 300 to 3400 leukocytes/mm^3. In all patients who had leukopenia and thrombocytopenia when treatment was stopped, counts returned to pretreatment levels or higher.

Other side effects noted were edema in two patients, nausea in one patient, and diarrhea in one patient. These data do not support the suggestion that dibromomannitol may induce gastrointestinal toxic effects or dermatitis; nonetheless, this possibility does exist.

DISCUSSION

Because none of the 14 patients with blastic transformation of CML and none of the 12 patients with refractory CML experienced a true remission after treatment with dibromomannitol, it was considered inappropriate to pursue this study further. The toxic effects induced by dibromomannitol were chiefly myelosuppressive. Despite discontinuance of treatment when the leukocyte count fell to less than 4000 cells/mm^3 in some instances, the toxicity was severe enough to be life threatening with leukocyte counts of 300 cells/mm^3.

We did not attempt to explore the usefulness of dibromomannitol in the management of CML in previously untreated patients.

LIST OF COMPOUNDS

Azathioprine: NSC-39084; CAS reg. No. 446-86-6; purine, 6-[(1-methyl-4-nitroimidazol-5-yl)thio]-, monohydrate; Imuran

Busulfan: NSC-750; CAS reg. No. 55-98-1; 1,4-butanediol, dimethanesulfonate; Myleran

Dibromomannitol: NSC-94100; CAS reg. No. 488-41-5; mannitol, 1-6-dibromo-1,6-dideoxy-, D-; myelobromol

Mannitol myleran: NSC-37538; CAS reg. No. 1187-00-4; mannitol, 1,6-dimethanesulfonate, D-

6-Mercaptopurine: NSC-755; CAS reg. No. 6112-76-1; purine-6-thiol, monohydrate; 6-MP; Purinethol

Prednisone: NSC-10023; CAS reg. No. 53-03-2; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

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Treatment of Advanced Gastrointestinal Cancer With Emetine (NSC-33669) [1,2,3]

Charles G. Moertel, Allan J. Schutt, R. G. Hahn, and R. J. Reitemeier [4,5]

SUMMARY

Twenty-five patients with metastatic gastrointestinal carcinoma were treated with emetine utilizing two dose regimens: a total of 10 mg/kg given in divided doses over 10 consecutive days, or a total of 15 mg/kg given in divided doses at 4-day intervals over 60 days. Observed toxic effects were nausea, vomiting, elevations of serum creatine kinase levels, and dose-related muscle weakness and electrocardiographic changes. In regard to antineoplastic therapeutic activity, only one patient showed incomplete and evanescent tumor regression. Emetine seems to be of no significant value in the management of advanced gastrointestinal cancer.

[Cancer Chemother Rep 58:229–232, 1974]

Emetine has been employed to treat a variety of human ills for several centuries. Its only accepted role in modern therapeutic efforts has been for certain parasitic infections, particularly amebiasis as described by Vedder in 1912 (1). In 1918 and 1919, reports appeared alleging the effectiveness of emetine in the treatment of cancer in man (2,3). Indeed, Van Hoosen (3) noted responses in 35 of 100 patients with advanced malignant disease. Surprisingly, this glowing report was not pursued. Interest in emetine as an antineoplastic agent, however, was recently revived by its demonstration of activity in several animal tumor screens including the L1210 and P388 mouse leukemias, and the B16 melanoma.[6] Subsequent studies in man with emetine or its derivatives have, as yet, been sparse, but objective responses have been reported with dihydroemetine [7] in the treatment of patients with adenocarcinoma of the rectum (4), Hodgkin's disease (4), chronic myelogenous leukemia (5), and bladder carcinoma (6), and with emetine in the treatment of patients with bronchogenic carcinoma (7) and teratocarcinoma (7).

Two phase I (dose and toxicity) cancer chemotherapy studies of emetine have been reported (7,8). Toxic reactions were primarily skeletal and cardiac myopathy, and when the drug was given at large doses, these effects could be disabling and sometimes fatal. Mastrangelo et al (8) concluded that the maximum tolerable total dose was 10 mg/kg. Panettiere and Coltman (7) suggested a higher dose, but stated that the toxic effects of eme-

[1] Received Aug 9, 1973; accepted Sept 21, 1973.

[2] Supported by contract NIH 70-2066 from the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3] Emetine: CAS reg. No. 316-42-7; emetine, hydrochloride. Supplied by Cancer Therapy Evaluation, DCT, NCI.

[4] Mayo Clinic and Mayo Foundation, Rochester, Minn.

[5] *Reprint requests to:* CG Moertel, MD, Mayo Clinic, Rochester, Minn 55901.

[6] Livingston RB, and Carter SK. Clinical brochure: emetine hydrochloride (NSC-33669). Prepared by the NCI, Bethesda, Md, 1969, 39 pp.

[7] Dihydroemetine: NSC-129414; emetine, 2,3-didehydro-, dihydrochloride, (±)-.

tine may become prohibitive above a total dose of 15 mg/kg.

Our investigation was undertaken to evaluate the therapeutic effectiveness of emetine in advanced gastrointestinal cancer utilizing two dose regimens: 1 mg/kg for 10 consecutive days (total dose, 10 mg/kg), and 1 mg/kg every 4 days (total dose, 15 mg/kg).

MATERIALS AND METHODS

Patient Selection

Twenty-five patients were entered into the study with histologically confirmed unresectable metastasis from primary carcinomas of gastrointestinal origin. The specific neoplasms are listed in table 1. Sixteen patients were men and nine were women. The mean and median age was 59 years with a range of 42–78 years. All were ambulatory outpatients, and all were maintaining a reasonable state of nutrition prior to entry. None had any clinical manifestations of heart disease before treatment. One patient, however, had electrocardiographic evidence of a ventricular conduction disturbance, and one patient had suggestive evidence of an old myocardial infarction.

Each patient had a measurable area of known malignant disease that served as an objective indicator of the patient's response to therapy. Discrete lesions on chest roentgenograms, cutaneous lesions, or palpable masses that were clearly measurable with a ruler or caliper were considered acceptable. If hepatomegaly was used as an indicator lesion, it was required that hepatic metastasis be proven by biopsy and that a clearly defined liver-edge extend at least 5 cm below the costal margin on quiet respiration. Lesions demonstrable only by contrast roentgenography or lesions for which size could only be estimated (eg, pelvic masses) were not acceptable.

TABLE 1.—Site of primary malignant neoplasm in patients treated with emetine

Primary site	No. of patients
Colorectal	18
Hepatoma	2
Stomach	2
Anus	1
Gallbladder	1
Pancreas	1

Ten patients had had no previous cancer treatment with cytotoxic drugs. Fifteen patients had had previous chemotherapy, primarily with 5-fluorouracil [8] or a nitrosourea. Four patients had had previous radiation therapy. None had had either radiation therapy or chemotherapy for at least 1 month before entry into this study.

Treatment Methodology

Emetine was administered by deep subcutaneous (sc) injection. In 13 patients, the dose schedule was 1 mg/kg/day for 10 consecutive days (total dose, 10 mg/kg), the usual amebiasis regimen. In 12 patients, the dose schedule was 1 mg/kg every 4 days (maximum total dose, 15 mg/kg).

Prior to treatment, all patients had a complete physical examination and measurement of indicator lesions. Pretreatment studies included: complete blood cell counts; urinalysis; serum creatinine, bilirubin, glutamic oxaloacetic transaminase, alkaline phosphatase, and creatine kinase determinations; bone marrow examination; electrocardiogram (ECG); electromyogram; and a chest roentgenogram. These evaluations were repeated at 4 and 8 weeks after the start of therapy. On each treatment day the pulse and blood pressure were determined, and the patient was asked to perform a standard series of exercises to assess muscle strength. Patients treated on the 10-day regimen also had ECGs and creatine kinase determinations on alternate treatment days, and leukocyte and platelet counts twice weekly for 8 weeks after the start of therapy. Patients on the every-4-day schedule had these studies on each treatment day.

Evaluation of therapeutic results was conducted at 4 and 8 weeks after the start of therapy. An objective response was declared if there was a reduction of at least 50% in the product of the longest perpendicular diameters of the most clearly measurable area of known malignant disease which was chosen prior to entry as a primary indicator lesion. If hepatomegaly was the indicator lesion, it was required that there be a reduction of at least 30% in the sum of measurements below

[8] 5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8.

the xyphoid process and each costal margin at the midclavicular lines without any deterioration of liver function. There could be no increase in size of other areas of known malignant disease and no new lesions could appear.

RESULTS

All of the 13 patients on the 10-consecutive-day program completed their planned 10-mg/kg total dose. Of the 12 patients entered on the every-4-day regimen, seven were able to complete their planned 15-mg/kg total dose. Treatment of the remaining five patients was discontinued because of severe and disabling progression of their malignant disease after they had received total doses of 7, 7, 8, 8, and 11 mg/kg, respectively.

Toxicity

The toxic reactions to both regimens of emetine therapy are documented in table 2. Nausea and vomiting occurred in about one half of the patients. This was mild, transient, and did not significantly impair nutrition.

Symptomatic muscle weakness with declining performance on exercise tests was most frequent in patients on the every-4-day schedule, and was generally first apparent between 3 and 4 weeks after the initiation of therapy. It was impossible, however, to clearly distinguish weakness that occurred as a secondary effect of emetine from that which occurred as an effect of advancing malignant disease.

Elevations in creatine kinase levels were frequent in patients on each schedule and were usually observed on the last day of the 10-day regimen and during the 4th or 5th week of the every-4-day regimen. These changes could not be clearly correlated to electrocardiographic or electromyographic changes or to clinical assessment of muscle weakness.

TABLE 2.—Rate of occurrence of toxic reactions during emetine therapy

Toxic reaction	Dose regimen*	
	10-day course	Every-4-day course
Nausea and vomiting	7/13	5/12
Muscular weakness	2/13	6/12
Increased creatine kinase (>50%)	7/13	9/12
Electrocardiographic changes	4/13	9/12

*No. of patients with toxic reactions/total No. of patients treated.

Electrocardiographic changes were most frequent in patients on the every-4-day schedule and seemed to be mainly dose related. Six of the seven patients treated with the full 15-mg/kg dose showed distinct abnormalities. These abnormalities are listed in table 3. The most frequent changes noted were in repolarization and conduction. In no instance was the electrocardiographic abnormality associated with significant cardiac symptoms.

Electromyographic changes were minimal and consisted only of fibrillation observed in three patients. There was no evidence of hematologic toxic effects obtained from either peripheral blood counts or serial bone marrow examinations, nor was there any suggestion of hepatic or renal toxic effects.

Therapeutic Results

A single patient with cancer of the large bowel showed partial regression of malignant hepatomegaly at 4 weeks associated with symptomatic improvement and improvement of liver function test results. At 8 weeks, however, this patient showed gross progression of his malignant disease. Three patients were objectively stable at 4 and 8 weeks. The remaining 21 patients showed progressive advance of their cancer associated with increasing symptoms and general deterioration.

DISCUSSION

At the dose levels employed in this study, therapy with emetine seems to be clinically tolerable. In view of the disabling and occa-

TABLE 3.—Nature of electrocardiographic abnormalities observed in 13 patients during emetine therapy

Abnormality	No. of patients
T-wave changes	10
Depressed ST segment	3
Prolonged RT interval	3
Atrial conduction delay	2
Atrial tachycardia	4
Atrial fibrillation	1
Bigeminy	1

sionally fatal toxic effects observed by others, we would be hesitant to explore higher dose levels.

From the therapeutic standpoint, only one of our patients showed a partial and very transient antineoplastic effect; the treatment cannot be considered even remotely valuable for any of the patients. Among the 35 patients with measurable areas of malignant disease in the combined studies of Panettiere and Coltman (7) and Mastrangelo et al (8), tumor regressions were observed in two. However, the clinical value of this measured tumor shrinkage was questionable, since cerebral metastasis developed in both patients during therapy.

In regard to gastrointestinal cancer, we must concur with the general conclusion of Mastrangelo et al (8) that emetine is of little value as a neoplastic agent when used alone at tolerable doses.

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Chemotherapy for the Terminal Phase of Chronic Myelocytic Leukemia [1,2]

Donald M. Hayes, Rose Ruth Ellison, Oliver Glidewell, James F. Holland, and Richard T. Silver [3,4]

SUMMARY

The results of therapy for the terminal phase of chronic myelocytic leukemia (CML) are uniformly poor. The studies described in this paper were designed (*a*) to test the criteria developed by Karanas and Silver in 1968 for the definition of the terminal phase of CML and (*b*) to determine the efficacy of several regimens for the treatment of the terminal phase of CML. In a phase I study 30 patients were assigned at random to one of four treatment groups: (*a*) 6-mercaptopurine "high" (1200 mg/m² given intravenously [iv] twice weekly), (*b*) 6-mercaptopurine "low" (800 mg/m² given iv twice weekly), (*c*) 6-thioguanine plus azaserine "high" (80 mg/m² of 6-thioguanine plus 32 mg/m² of azaserine given orally daily), and (*d*) 6-thioguanine plus azaserine "low" (40 mg/m² of 6-thioguanine plus 16 mg/m² of azaserine given orally daily). The lack of response in the first six patients treated with 6-mercaptopurine and the excessive toxicity in the first six patients treated with 6-thioguanine plus azaserine "high" resulted in the abandonment of these treatment schedules. Two of the 18 patients treated with 6-thioguanine plus azaserine "low" had partial

[1] Received Oct 2, 1973; accepted Oct 25, 1973.

[2] 6-Thioguanine, cytosine arabinoside, BCNU, and vincristine were supplied by Cancer Therapy Evaluation, Division of Cancer Treatment, National Cancer Institute (NCI), National Institutes of Health (NIH). Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are listed in the List of Compounds at the end of the text.

[3] This study was done by the Acute Leukemia Group B; Operations Office (James F. Holland, chairman, and Oliver Glidewell). The following institutions and investigators participated in the study: Boston Floating Hospital for Infants and Children, New England Medical Center Hospital, Boston, Mass (Thomas F. Necheles); Bowman Gray School of Medicine, Winston-Salem, NC (Donald M. Hayes; CA-03927); Centre d'Onco-Hematologie, Geneva, Switzerland (Pierre Maurice); Coney Island Hospital, Brooklyn, NY (Joel M. Schwartz; CA-05923); Finsen Institute, Copenhagen, Denmark (Nis I. Nissen); Hematology and Oncology Station, Zurich, Switzerland (Georg Martz); University of Pretoria, H. F. Verwoerd Hospital, Pretoria, South Africa (Geoffrey Falkson); Medical College of Virginia, Richmond (John H. Moon [deceased]; CA-03735); Montreal General Hospital, Montreal, Canada (J. L. Hutchison); Mt. Sinai Hospital, New York, NY (Janet Cuttner; CA-04457); Massachusetts General Hospital, Boston (Robert Carey); New York Hospital, Cornell Medical Center, New York, NY (Richard T. Silver, chairman, Chronic Leukemia Subcommittee; CA-07968); Rhode Island Hospital, Providence (Louis A. Leone and Vishram B. Rege; CA-08025); Roswell Park Memorial Institute, Buffalo, NY (Rose Ruth Ellison; CA-02599); Royal Victoria Hospital, Montreal, Canada (Lorne Shapiro); Schweizerische Chemotherapiegruppe, Bern, Switzerland (Kurt W. Brunner and Paul Obrecht); Long Island Jewish Medical Center, New Hyde Park, NY (Arthur Sawitsky and David Prager); Maimonides Hospital of Brooklyn, NY (Norton D. Ritz; CA-05923); US Naval Hospital, San Diego, Calif (Eugene Lang); US Naval Hospital, Philadelphia, Pa, and Thomas Jefferson University Hospital, Philadelphia (Richard Burningham and Farid I. Haurani; CA-05462); University of Missouri, Columbia (Patrick H. Henry); Walter Reed Army Medical Center, Washington, DC (Johannes Blom); West Virginia University, Morgantown (John B. Harley; CA-05923). Supported by Public Health Service research grants (CA) from the NCI, NIH, Department of Health, Education, and Welfare.

[4] *Reprint requests to:* Acute Leukemia Group B Operations Office, Suite 3A-3B, 651 Elm St, Buffalo, NY 14203.

responses, three had improvement, and the rest had either no response or progressive disease. In a phase II study, 21 patients were treated with a combination of BCNU at a dose of 4.5 mg/m² and cytosine arabinoside at a dose of 30 mg/m² given iv every 3 hours for eight doses on Days 1, 5, 9, and 13. Treatment with this regimen resulted in only two partial responses, but there was sufficient antileukemic activity to warrant a further trial of a modified regimen. Forty-seven patients were then treated with BCNU at a dose of 4.5 mg/m² and cytosine arabinoside at a dose of 45 mg/m² given iv every 12 hours for eight doses on Days 1–4, 13–16, 25–28, and 37–40. The total response rate was 17% including three complete and five partial responses. Patients in the final study group (phase III) were treated as follows: cytosine arabinoside (45 mg/m²) and BCNU (4.5 mg/m²) were administered iv every 12 hours for eight doses, vincristine (2.0 mg/m²) was administered iv on Day 5, and prednisone (60 mg/m²) was administered orally daily on Days 5–9. Treatment courses were repeated up to three times. Of the 39 patients entered into the study, one had a complete response, four had partial responses, five had improvement, 13 had no response, and 14 had progressive disease despite therapy. None of the treatments described in this paper were successful enough to warrant recommendation for general use against the terminal phase of CML. However, these studies do validate the work of Karanas and Silver indicating that "terminal phase" CML is a recognizable entity and that patients with this form of the disease constitute a useful group for clinical trials of new antileukemic agents.

[Cancer Chemother Rep 58:233–247, 1974]

Although the prognosis for chronic myelocytic leukemia (CML) is generally better than that for the acute leukemias, there is a point in the natural progression of CML when the disease appears to be totally resistant to therapy. This phase has been called "terminal phase" (1), "blastic transformation" (2), "blast crisis" (3), and "accelerated phase" (4). Regardless of the name used, the clinical features of the disease at this time assume in many but not all patients the characteristics of acute myelocytic leukemia (AML) of adult life. Despite a paucity of documentation, chemotherapists agree that the terminal phase of CML is more resistant to therapy than is AML in adults.

Terminal phase CML is characterized by resistance to therapy with busulfan (5), dibromomannitol (6), radiation (7), and other measures that are effective earlier in the course of the disease. Occasional responses to 6-mercaptopurine (3), methotrexate (8), and other drugs (9) have been reported, but the results of single-drug treatment with conventional agents have generally been discouraging.

In this paper, we describe the experiences of the Acute Leukemia Group B (ALGB) in the treatment of terminal phase CML from 1964 to the present. The experiences are described as phase I, II, and III studies.

PHASE I STUDY

Combination chemotherapy for the acute leukemias (10) and lymphomas (11) has proved superior to single-drug chemotherapy. Theoretical bases for this superiority have been demonstrated in a number of animal model systems. Tarnowski and Stock (12) noted that in mice bearing the RC mammary carcinoma treatment with azaserine or 6-diazo-5-oxo-L-norleucine combined with 6-mercaptopurine caused a significantly greater number

of tumor regressions than did the same drugs given alone; this demonstrated a synergistically increased inhibitory effect on tumor growth. Sartorelli and LePage (13) noted that Ehrlich ascites carcinoma cells are sensitive to combinations of azaserine and 6-thioguanine. The most effective dose schedule was 0.2 mg/kg of azaserine plus 0.5 mg/kg of 6-thioguanine given twice daily. Simultaneous administration of the drugs was more effective than alternate administration. When 6-thioguanine was used in combination and administered parenterally to patients who had solid tumors, the dose could be increased to as much as 1.7 mg/kg twice daily for 7 days before prohibitive vomiting and diarrhea occurred (14). Additional screening studies showed that treatment with the azaserine-6-thioguanine combination was more effective against L1210 leukemia than was treatment with either agent given alone (15).

In view of the data cited, the ALGB began clinical trials of a 6-thioguanine-azaserine combination and of intravenously (iv) administered 6-mercaptopurine in patients with terminal CML.

Materials and Methods

Patients from the participating institutions were selected on the basis of criteria described by Karanas and Silver (1) and adopted by the ALGB. For each patient, a previously established histologic and biochemical diagnosis of CML was required. Additional requirements for admission to the study are listed in table 1.

Thirty patients were admitted to the study: 16 were male and 14 were female. The median age was 53 years (range, 6–84 years). All but one of the patients had been treated with at least one agent, and several had been treated with more than one. Twenty-eight patients had been treated with busulfan, seven with 6-mercaptopurine, two with prednisone, two with cyclophosphamide, two with radioactive phosphorus (^{32}P), four with radiation therapy (not otherwise specified), and one each with splenic irradiation, dibromomannitol, and uracil mustard. The median white blood cell count (wbc) at entry into the study was 42,000 cells/mm^3, the median platelet count was 24,000 platelets/mm^3, and the median hemoglobin value was 7.8 g/100 ml.

Initially, patients entering the study were assigned at random to one of four treatment groups:

(*a*) Treatment "A, high": 6-mercaptopurine at a dose of 1200 mg/m^2, given iv twice weekly;

TABLE 1.—Requirements for admission to the study

(1) Peripheral blood blasts and promyelocytes ≥30% at any time *or*

(2) Either (*a*) or (*b*)—whichever came first—*plus* one of the conditions listed under (3)
- (*a*) Completion of two courses of "conventional" treatment
- (*b*) Survival of at least 12 months after diagnosis

(3) At least one of the following conditions in the absence of hemorrhage or toxicity
- (*a*) Peripheral blood blasts and promyelocytes ≥20%
- (*b*) Hemoglobin value ≤9.0 g/100 ml
- (*c*) Platelet count decreased from >100,000 platelets/mm^3 to <100,000 platelets/mm^3 during a conventional course of treatment
- (*d*) An increase of the wbc after a conventional course of treatment to either 50,000 cells/mm^3 or more than twice the count at the start of the course of treatment

(4) No chemotherapy for 2 weeks preceding experimental therapy

(5) No splenic or spray irradiation during experimental therapy. Palliative therapy through one port permissable during study if clinically indicated.

(6) No elective corticosteroid therapy during experimental therapy

(7) No exposure to drugs used in this study within 3 weeks prior to the study

(*b*) Treatment "A, low": 6-mercaptopurine at a dose of 800 mg/m², given iv twice weekly;

(*c*) Treatment "B, high": 6-thioguanine at a dose of 80 mg/m²/day, given orally, plus azaserine at a dose of 32 mg/m²/day given orally;

(*d*) Treatment "B, low": 6-thioguanine at a dose of 40 mg/m²/day, given orally, plus azaserine at a dose of 16 mg/m²/day, given orally.

Treatment was to be continued for at least 42 days unless progressive disease supervened. If a response occurred, treatment was to be continued until the patient had a relapse. Patients were to be "crossed over" to the opposite drug regimen after 42 days if no response was achieved, after 70 days if at least a partial response was not achieved, or after relapse from a response status. At the time of "cross over" to the opposite treatment, patients were to be reassigned to "high"- or "low"- dose treatment according to the randomization cards.

Study parameters and the frequency of their determinations are listed in table 2.

Results

Most of the elaborate study plan outlined previously did not come to pass. Treatments "A, high" and "B, high" soon proved to be formidably toxic and they were abandoned. Treatment "A, low" was soon found to be ineffectual and it was abandoned also. Thus, most of the patients were given treatment "B, low."

Ratings for response to therapy are shown in table 3. Responses and toxicity are presented according to treatment group.

Treatment "A, high" (6-mercaptopurine, 1200 mg/m², given iv twice weekly).—Only two patients were treated in this group. Both experienced severe toxicity without demonstrable benefit. Toxicity in one patient, a 31-year-old woman, was mainly hematopoietic with leukopenia (wbc of 600–1400 cells/mm³) persisting 70 days after only three doses of the drug. The other patient, a 78-year-old man, ex-

TABLE 2.—Study parameters

Parameter	Schedule
Peripheral blood: complete blood cell count and reticulocyte count	Twice weekly for 2 weeks; once weekly thereafter
Bone marrow examination	At beginning of treatment; at 4, 8, and 12 weeks after start of treatment; and as clinically indicated
Blood chemical values: BUN, uric acid, and bilirubin	Before start of treatment; once weekly thereafter
Urinalysis	Weekly for 2 weeks; then biweekly
Measurable lesions:	Measurements to be recorded twice before treatment and then at each visit
Spleen—to be measured in cm perpendicular to the left costal margin at the midclavicular line on deep inspiration	
Liver—to be measured in the same manner as the spleen from the right costal margin	
Lymph nodes—to be measured with vernier calipers in the two greatest dimensions	

TABLE 3.—Rating of response to therapy for patients with terminal phase CML

Type of response	Hematologic status					Physical status—spleen (cm below left costal margin)	Performance status
	Wbc (cells/mm³)	Differential count	Hemoglobin (g/100 ml)	Hematocrit (%)	Platelet count (platelets/mm³)		
Complete	≤15,000	Promyelocytes and myeloblasts ≤3%	≥12 for male patients ≥11 for female patients	≥37 for male patients ≥34 for female patients	≥150,000	≤2	No symptoms
Partial	≥15,000 but ≤50,000 or ≤50% of wbc at the start of therapy	Promyelocytes and myeloblasts >3% but <20%	≥10 but <12 for male patients <11 for female patients	≥30 but <36 for male patients <33 for female patients	From ≤100,000 to ≥100,000 but <150,000	>2 but <12	Symptoms of disease with normal or limited activity but <50% of patient's normal waking hours spent in bed
No significant response	No significant change in any parameter						

perienced severe nausea, vomiting, and bloody diarrhea after each dose of the drug; these symptoms persisted for 2 weeks until treatment was modified to the lower dose.

Treatment "A, low" (6-mercaptopurine, 800 mg/m², given iv twice weekly).—Five patients were included in this group. None experienced measurable improvement. Toxicity was moderate and occurred mainly in the form of hematopoietic depression. The median initial wbc in this group was 11,100 cells/mm³, the median hemoglobin value was 8.5 g/100 ml, and the median platelet count was 46,000 platelets/mm³. The median nadir values reached after treatment were 8600 cells/mm³, 3.75 g/100 ml, and 8000 platelets/mm³, respectively. These values made it apparent that this dose level was myelosuppressive despite the absence of a therapeutic response. Although this patient group was too small for firm conclusions, it was the ALGB group consensus that this treatment was so manifestly ineffective that it should be abandoned.

Treatment "B, high" (6-thioguanine, 80 mg/m²/day, given orally, plus azaserine, 36 mg/m²/day, given orally).—One of the six patients treated with this regimen died after only 1 day. The remaining five patients were treated for 4, 5, 15, 19, and 35 days. Despite the brevity of these trials, the nature of terminal phase CML is such that any effective treatment must be rapid in action. Thus, all five of these patients were considered evaluable. None achieved a true response although decreases in wbc counts, splenomegaly, and symptoms were noted in two.

Toxic effects included one instance each of cholestatic jaundice and one instance of profound leukopenia.

Treatment "B, low" (6-thioguanine, 40 mg/m²/day, given orally, plus azaserine, 16 mg/m²/day, given orally).—One of the 18 patients in this treatment group was lost to followup after 17 days and was excluded from evaluation. All remaining patients were treated from 10 to 110 days.

Detailed therapeutic and toxicity data for responding patients are recorded in table 4.

Although two partial responses were achieved, there were no complete responses. In addition, the brevity of these responses was striking (median duration, 35 days).

Discussion

The criteria for identifying terminal phase CML (1) were strengthened by these observations. Even those patients experiencing little or no toxicity died within the study period, a fate predicted by the criteria.

6-Mercaptopurine was selected for study in the form for iv administration because "occasional responses" to orally administered therapy at lower doses had been reported (3). Although toxicity was achieved with the doses used and although the patient groups were small, there were no indications from these data that iv administered 6-mercaptopurine was effective against terminal phase CML.

Although the toxicity of treatment with the high-dose combination of azaserine and 6-thioguanine precluded its continuation, the low-dose combination was relatively well toler-

TABLE 4.—Clinical and laboratory data for patients responding to treatment with the low-dose 6-thioguanine-azaserine combination

Patient No.	Wbc (× 10³ cells/mm³)		Platelet count (× 10³ platelets/mm³)		Spleen (cm below left costal margin)		Toxicity*			Response†	Duration of treatment (days)
	Initial value	Best value achieved	Initial value	Best value achieved	Initial value	Best value achieved	Hematologic	Gastrointestinal	Other		
1	43.3	9.4	16.0	120.0	6	0	3	0	0	PR	62
2	21.0	5.7	16.0	64.0	‡	—	0	0	0	PR	110

*0 = No toxicity; 1= mild; 2 = moderate (requiring a change in drug dose); 3 = severe (requiring discontinuance of treatment with the drug); 4 = severe (life-threatening or fatal).

†PR = partial response.

‡Previous splenectomy.

ated. However, only slight antileukemic effect was demonstrated.

We would like to emphasize that terminal phase CML is a rigorous testing ground for new therapies. The disease at this point is so rapidly and inexorably fatal that a new treatment must be not only very effective but also rapid in its effect. These treatments obviously did not meet these requirements.

PHASE II STUDY

Because iv administered 6-mercaptopurine and azaserine plus 6-thioguanine had little effect on terminal phase CML, the effect of another treatment (cyclically administered cytosine arabinoside combined with BCNU) against this variety of leukemia was studied.

Impressive laboratory results have been reported by Skipper, Schabel, and Wilcox for L1210 leukemia (16). BDF_1 mice given 10^7 leukemic cells iv have a uniform 100% mortality rate within 4 days. When these animals were given cytosine arabinoside at a dose of 15 mg/kg iv every 3 hours for 24 hours with courses repeated at 4-day intervals, the mean survival time was extended to 18 days, but there were no "long-term" (45-day) survivors. Treatment with BCNU alone in a similar fashion at a dose of 1.5 mg/kg caused extension of the mean survival time to 30 days with one of ten animals surviving 45 days. Combination treatment with 15 mg/kg of cytosine arabinoside and 1.0 mg/kg of BCNU every 3 hours for 24 hours (repeated for a total of four courses) yielded a mean survival time of more than 45 days with ten of ten animals surviving 45 days. In addition, treatment with this combination caused no deaths in nonleukemic animals. Treatment with a combination of cytosine arabinoside and cyclophosphamide yielded similar results in the same test system.

Cytosine arabinoside has been used alone by the ALGB in infusions of 1, 4, 12, and 24 hours (17). Therapeutic benefits in terminal phase CML were not impressive. BCNU has not been tried alone in the treatment of terminal phase CML. Dose schedules for iv administration of 250 mg/m² singly, 125 mg/m² for 3 successive days, and 90 mg/m²/week for 6 weeks were found to be tolerable (18).

Two treatment programs are described in the following sections. The first, program A, is a direct trial of the optimum dosages observed in experiments with L1210 leukemia. The second, program B, is a time-modified cyclic treatment allowing for the difference in generation time between L1210 cells and human leukemia cells.

Treatment Programs

Program A consisted of treatment with BCNU at a dose of 4.5 mg/m² and cytosine arabinoside at a dose of 30 mg/m² given iv every 3 hours for eight doses on Days 1, 5, 9, and 13. Program B consisted of the same dose of BCNU given iv every 12 hours for eight doses on Days 1–4, 13–16, 25–28, and 37–40, and cytosine arabinoside given at the same time intervals but with the dose increased to 45 mg/m². All patients received prophylactic and therapeutic platelet infusions throughout the study.

Eligibility for Study

A previously established diagnosis of CML was required for admission to the study. Other requirements are listed in table 1.

Patients

Program A.—Twenty-one patients were entered into the initial program, the direct trial of the optimum cyclic therapy for L1210 leukemia. Eight patients were men and 13 were women. The median age was 52 years. The median time that had elapsed from the time of original diagnosis was 30 months. Previous therapies included busulfan (17 patients), dibromomannitol (four patients), 6-mercaptopurine (four patients), ^{32}P (one patient), daunorubicin (one patient), splenic irradiation (one patient), and methyl-GAG (one patient).

Program B.—Forty-seven patients were entered into the study. Twenty-five patients were women and 22 were men. The median age was 52.5 years. The median time from the original diagnosis to the onset of the terminal phase was 28 months. All patients had received previous therapy: 41 with busulfan,

19 with 6-mercaptopurine, nine with dibromomannitol, six with splenic irradiation, four with cyclophosphamide, four with vincristine, six with prednisone, three with methotrexate, two with ^{32}P, and one each with daunorubicin, chlorambucil, thioTEPA, melphalan, and uracil mustard.

Studies prior to the start of therapy included the items described in table 2. The cases of patients in program A were followed with daily complete blood cell counts and reticulocyte and platelet counts during active therapy (14 days) and twice weekly thereafter. Bone marrow examination was repeated on Days 4, 8, 14, and 21. Patients in program B were studied in a similar manner except that bone marrow examinations were done on Days 0, 12, 24, 36, and 50 and whenever necessary for clarification of toxic or therapeutic effect. At any time that the wbc fell to 1000–2000 cells/mm^3, therapy was continued at full dose if the marrow remained normocellular or hypercellular and "blastic." If the wbc fell to less than 1000 cells/mm^3 and the marrow remained hypercellular, therapy was continued at one half of the dose but at the same time intervals as before. If the wbc fell to less than 2000 cells/mm^3 and the marrow was hypocellular, therapy was discontinued until marrow regeneration occurred.

Results

Responses were evaluated according to criteria adopted by the ALGB as shown in table 3.

Program A was the study performed first. In the 21 patients entered into the study, one complete and one partial response occurred. Only the data obtained for these two responding patients are given in table 5. However, even in those patients who did not have complete or partial responses there was often a striking degree of antileukemic activity. This was most often a precipitous fall in wbc or a rapid reduction in spleen size. This evidence of profound antileukemic effect led the group to proceed with program B.

The results of therapy with program B are shown in table 6. Of the 47 patients entering the study, 45 were considered evaluable (two were lost to followup). Three complete and five partial responses were achieved. In addition, in 12 patients there was measurable improvement although all criteria necessary for a response to be considered complete or partial were not met. Five of these patients regained normal or near normal marrows but remained leukopenic or thrombocytopenic. Thus, the overall remission rate of 18% included only 7% complete responses and 11% partial responses and none of the patients with improvement who did not completely qualify for remission status.

Total survival time from the start of therapy ranged from 11 to 195 days with a median of 35 days. In nonresponding patients, the median survival time was 26 days. In responding patients, all of whom received maintenance therapy, the median survival time was 53 days.

In ten of the patients in program A no toxicity occurred. The major toxic effect was hematopoietic depression, which was fatal or near-fatal in three patients. One patient had fatal sepsis, but it was not clear whether or not this was drug-related. In patients in program B, the toxic effects were similar. However, the frequency was less since no toxicity occurred in 20 patients and life-threatening or fatal toxicity developed in only seven of the 47 patients (marked hematologic toxicity in four patients and overwhelming sepsis in three). The remaining instances of toxicity were thought to be of acceptable magnitude although fatal hemorrhage would surely have been more frequent were it not for the constant availability of platelet supplements.

Discussion

In most varieties of human leukemia, a response rate such as the one reported here would hardly be regarded as exciting. However, the terminal phase of CML is so highly resistant to treatment that any type of therapy yielding a measurable response rate is worthy of comment and further study. Foley, Bennett, and Carbone (4), using a combination of vincristine, prednisone, methotrexate, and 6-mercaptopurine, were able to obtain one complete and two partial responses in treating 13 patients with terminal phase CML, but these responses were gained at the cost of severe toxicity with four drug-related deaths occur-

TABLE 5.—Clinical details for patients responding to program A treatment

Patient No.	Age, sex	Months from diagnosis	Previous treatment	Spleen (cm below left costal margin)		Hematologic values: Wbc (× 10^3 cells/mm^3)			Hematologic values: Platelet count (× 10^3 platelets/mm^3)			Response*	Duration of treatment (days)
				Before treatment	After treatment	Before treatment	Nadir	Best	Before treatment	Nadir	Best		
1	64,M	Unknown	Unknown	10	0	84.0	2.0	7.9	33.0	8.0	245.0	CR	154
2	35,M	21	Busulfan	1	3	87.5	6.1	6.8	110.0	20.0	105.0	PR	63

*CR = complete response; PR = partial response.

TABLE 6.—Clinical details for patients responding to program B treatment

Patient No.	Age, sex	Months from diagnosis	Previous treatment	Spleen (cm below left costal margin)		Hematologic values						Response*	Duration of treatment (days)
						Wbc (× 10^3 cells/mm^3)			Platelet count (× 10^3 platelets/mm^3)				
				Before treatment	After treatment	Before treatment	Nadir	Best	Before treatment	Nadir	Best		
1	26,F	22	Busulfan, 6-mercaptopurine, daunomycin, vincristine	3.5	0.5	0.35	0.5	4.5	20.0	5.0	71.0	PR (63 days)	89
2	39,M	58	Busulfan, 6-mercaptopurine	6	0	24.8	0.4	4.5	73.0	9.0	110.0	PR	85
3	51,F	8	Busulfan	8	2	91.2	0.2	5.3	16.0	3.0	250.0	CR	53
4	44,M	42	Busulfan, 6-mercaptopurine	0	0	24.0	0.65	5.7	79.0	3.0	276.0	CR	195
5	29,M	15	Dibromomannitol, busulfan	10	1	43.0	0.6	6.1	15.0	2.0	261.0	PR	70
6	45,M	48	Busulfan, 6-mercaptopurine	13	0	225.0	2.5	7.2	38.0	6.0	92.0	PR	82
7	61,M	24	Dibromomannitol	2	0	6.2	1.0	6.2	94.0	20.0	340.0	CR	85
8	67,F	60	Busulfan	4	0	128.7	23.5	23.5	43.0	10.0	63.0	PR	16

*PR = partial response; CR = complete response.

ring. Garson, Burgess, and Stanley (19), using smaller doses of the same preparations, were able to obtain complete marrow remission in a single case. Only occasional responses have been reported with a variety of other agents including methotrexate (8), methyl-GAG (9), and 6-mercaptopurine (10).

The achievement of response with two agents that had previously shown negligible activity when used alone against CML tends to validate the laboratory demonstrations of Skipper, Schabel, and Wilcox (16) concerning cyclic administration versus single-dose or continuous administration. Although the combination of cytosine arabinoside and BCNU given cyclically had definite antileukemic effect, its usefulness in patients with terminal phase CML is limited because of the decreased marrow reserve of these patients. In many instances in our study, it was possible to eradicate the leukemic cells from the marrow, but in the ensuing time interval (the period of waiting for marrow regeneration) there were many difficulties. The ALGB experience with this same cyclic combination against acute leukemia was similar (21).

PHASE III STUDY

A high rate of response (30%) to treatment with a combination of vincristine and large doses of prednisone in patients with terminal phase CML was reported by Canellos et al (22). Since this treatment and the cyclic cytosine arabinoside plus BCNU treatment (23) hypothetically exert their antileukemic effect by differing mechanisms and since their toxicities are presumably not additive, the ALGB instituted the study reported here. Its purpose was to determine whether the two regimens, each having moderate activity separately, would show additive or synergistic effects against terminal phase CML.

Materials and Methods

Thirty-nine patients were entered into this phase III study. Twenty-five patients were men and 14 were women. The median age was 41 years and the median time that had elapsed from the time of original diagnosis of CML to the onset of the terminal phase was 15 months. All patients had had one or more forms of therapy for CML, including busulfan, dibromomannitol, splenic irradiation, 6-mercaptopurine, corticosteroids, hydroxyurea, ^{32}P, vincristine, methotrexate, melphalan, and splenectomy.

All patients had a previously established diagnosis of CML and met the criteria for presence of the terminal phase listed in table 1. Any patient receiving corticosteroids for 6 weeks or more could be entered into the study and continue to receive steroids at a dose of 20 mg/day of prednisone or its equivalent.

Drugs for induction therapy were given as follows:

(*a*) BCNU was given iv at a dose of 4.5 mg/m^2 every 12 hours for 4 days (eight doses). Repetitive courses were given on Days 13–16, 25–28, and 37–40, if indicated.

(*b*) Cytosine arabinoside was given iv at a dose of 45 mg/m^2 every 12 hours for 4 days (eight doses); each injection followed an injection of BCNU. Repetitive courses were given on the same daily schedule as indicated in (*a*).

(*c*) Vincristine was given iv as a single dose of 2.0 mg/m^2 on Day 5 of each cycle, ie, on Days 5, 17, 29, and 41.

(*d*) Prednisone was given orally at a single daily dose of 60 mg/m^2 for 5 days beginning on the day of vincristine injection and repeated each cycle. The dose was calculated to the nearest 5 mg. Prophylactic antacids were given during the 5 days of prednisone therapy.

Hematologic and clinical evaluations were performed immediately before each course of therapy. No provision was made for dosage modification during the induction phase in response to thrombocytopenia, but investigators were advised to use platelet supplements generously and not to give this therapy if platelet supplements were not available. Laboratory studies performed before and during therapy are outlined in table 2.

Bone marrow examination was performed before the start of each course of therapy and at any time that the peripheral blood wbc was less than 2000 cells/mm^3 or the granulocyte count was less than 1000 cells/mm^3. If marrow aplasia or hypoplasia occurred, therapy was

suspended temporarily until regeneration occurred. Whenever remission status was achieved, maintenance therapy was begun. If a partial remission status was present prior to any course of therapy, that course was given. If the next marrow examination preceding another course of therapy showed that the marrow was unchanged, it was assumed that this was the maximum benefit achievable and maintenance therapy was begun. Four complete courses of therapy without response were judged as a treatment failure.

Maintenance therapy consisted of cytosine arabinoside at a dose of 30 mg/m^2 given subcutaneously weekly plus 6-thioguanine at a dose of 2.5 mg/kg/day given orally. Dosages for maintenance therapy were modified downward as necessary in accordance with changes in blood cell counts.

Results

The criteria for judging response are shown in table 3. Patients who experienced measurable significant improvement in at least one parameter but who did not achieve all criteria necessary for partial or complete response were judged to have had "improvement" but not a full response.

Detailed clinical and hematologic data concerning all patients who had a favorable response to therapy are shown in table 7. Although 39 patients were entered into study, two were subsequently dropped because it was found that they did not meet the diagnostic criteria for terminal phase CML. Of the 37 remaining patients, 11 died before receiving one full course of treatment, three had progressive disease despite therapy, 13 had no response, and ten had some degree of response (one patient had a complete response, four had partial responses, five had definite improvement although the improvement was not sufficient for the responses to qualify as full responses). Thus, the true overall response rate was 5 of 37 or 13.5%.

The median survival time for the entire group of patients was 58 days from the start of therapy. For responding patients, the median survival time was 97 days; for nonresponding patients, it was 49 days. This suggests some advantage for responding patients, but the groups are too small for the difference to be significant.

The major toxic effect was hematopoietic depression, which was severe in six patients, moderate in nine, and mild in three. Four of the patients in whom toxicity was severe died; death was judged to be drug-related in all. Gastrointestinal toxicity occurred in four patients and consisted of cholestatic jaundice in one patient and diarrhea in three patients. Neurologic toxicity occurred in five patients, all of whom suffered from peripheral neuropathy.

Discussion

It is obvious that this cyclic combination of drugs was neither additive nor synergistic in terms of antileukemic effect. Canellos and Carbone (22) reported 30% total responses in 30 patients, and the study of the ALGB yielded a 17% response rate in 45 patients. The reasons for the differences in response rates to these two therapies were not elucidated by the present study.

Lampkin, Nagao, and Mauer (24) suggest that the sequence of drug administration may be an important determinant of drug effect in the treatment of leukemia. Corticosteroids suppress the entry of leukemic cells into DNA synthesis. Cytosine arabinoside is an active inhibitor of DNA synthesis and has also been noted to exert a "recruitment" effect, causing cells to become more synchronous in their mitotic cycle. Vincristine arrests cells in mitosis. BCNU is apparently not phase-specific in its activity and it is actively cytotoxic in all phases of the mitotic cycle. Considering these data, we thought it most rational to give the four drugs in this sequence: cytosine arabinoside, BCNU, vincristine, and prednisone. Our results do not support the theory. Although this drug combination has some antileukemic effect in the terminal phase of CML, certain factors (as yet unknown) prevent it from producing the hoped-for frequency of response.

LIST OF COMPOUNDS

Azaserine: NSC-742; CAS reg. No. 115-02-6; serine, diazoacetate (ester), L-

BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-

TABLE 7.—Clinical data for patients responding to cyclic therapy

Patient No.	Age, sex	Months from diagnosis	Hematologic values						Response*	Duration of treatment (days)
			Wbc (× 10^3 cells/mm^3)		Platelet count (× 10^3 platelets/mm^3)		Spleen (cm below left costal margin)			
			At start of treatment	Best	At start of treatment	Best	At start of treatment	Best		
1	?,F	?	10.0	8.0	50.0	57.5	1	0	PR	107
2	37,F	36	31.0	5.8	18.0	90.0	3	0	PR	176
3	49,F	60	33.2	4.7	52.0	80.0	9	0	PR	103
4	64,M	Unknown	84.0	7.9	33.0	245.0	10	0	CR	154
5	35,M	21	87.5	6.8	110.0	105.0	1	0	PR	63

*PR = partial response; CR = complete response.

Busulfan: NSC-750; CAS reg. No. 55-98-1; 1,4-butanediol, dimethanesulfonate; Myleran

Chlorambucil: NSC-3088; CAS reg. No. 305-03-3; butyric acid, 4-[*p*-[bis(2-chloroethyl)amino]phenyl]-

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan

Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride

Daunorubicin: NSC-83142; CAS reg. No. 23541-50-6; rubidomycin; daunomycin (NSC-82151)

6-Diazo-5-oxo-L-norleucine: NSC-7365; DON

Dibromomannitol: NSC-94100; CAS reg. No. 488-41-5; 1,6-dibromo-1,6-dideoxy-D-mannitol; Myelobromol

Hydroxyurea: NSC-32065; CAS reg. No. 127-07-1

Melphalan: NSC-8806; CAS reg. No. 3223-07-2; alanine, 3-[*p*-[bis(2-chloroethyl)amino]phenyl]-, monohydrochloride, L-

6-Mercaptopurine: NSC-755; CAS reg. No. 6112-76-1; purine-6-thiol, monohydrate; 6-MP

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-; amethopterin

Methyl-GAG: NSC-32946; CAS reg. No. 24968-67-0; guanidine, 1,1'-[(methylethanediylidene)dinitrilo]di-, dihydrochloride, monohydrate; methylglyoxal-bis-(guanylhydrazone)

Prednisone: NSC-10023; CAS reg. No. 53-03-2; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-

6-Thioguanine: NSC-752; CAS reg. No. 1832-72-0; purine-6-thiol, 2-amino

ThioTEPA: NSC-6396; CAS reg. No. 52-24-4; phosphine sulfide, tris(1-aziridinyl)-; triethylenethiophosphoramide

Uracil mustard: NSC-34462; CAS reg. No. 66-75-1; uracil, 5-[bis(2-chloroethyl)amino]-

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

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Recurrent Masses at Sites of Prior Krebiozen® (NSC-8752) Injections [1,2]

David C. Stolinsky and Joseph R. Bateman [3,4]

Krebiozen® is a substance with no demonstrable anticancer activity. It has been banned from interstate shipment (1). This report describes an interesting result of its administration.

CASE REPORT

A 28-year-old man noted a mass on the calf of his right leg. The mass was excised widely and diagnosed as embryonal rhabdomyosarcoma. Two months later, three rib metastases were treated with radiation therapy. A hilar and superior mediastinal mass responded to further radiation therapy. However, 1 month later the patient was admitted to the hospital with multiple subcutaneous masses, generalized adenopathy, and hepatosplenomegaly. These masses responded transiently to cyclophosphamide [5] but then very rapidly increased in size once more. In addition, a striking superior vena cava syndrome appeared. Treatment with a combination of vinblastine and vincristine [6] was given for 3 days. During the next week, all masses decreased in size and the superior vena cava syndrome subsided. Leukopenia (white blood cell count, 1500 cells/mm^3), jaw pain, obstipation, numbness and weakness of the extremities, and alopecia developed. Two weeks after the start of therapy, all masses disappeared. Three-day courses of medication (following a Western Cooperative Cancer Chemotherapy Group protocol) were repeated as tolerated. Therapy was stopped after 5 months, and the patient's disease remained in complete remission for another 63 months.

At one clinic visit during this time (5 years after the cessation of therapy), a hard, warm, tender mass measuring 12 cm × 7 cm was felt in the left quadriceps muscle. Two 1-cm lymph nodes were felt in the left inguinal area, and a smaller mass was felt in the right quadriceps muscle as well. The patient assured the physician that there was no need for concern, because these masses appeared each time he had a respiratory tract infection and then disappeared once more. The patient's prediction was borne out when the masses in his thighs and lymph nodes disappeared spontaneously over the next 2 weeks. The results of serum protein electrophoresis were normal at this time. On further questioning, the patient revealed that 5 years earlier (before the start of chemotherapy) he had journeyed to Chicago, where he received daily injections of Krebiozen® into the anterior muscles of both thighs for 1 week. The disease progressed despite this "treatment" and the patient returned to care of his own physicians.

After 68 months in complete remission (63 months after discontinuing therapy), the patient had bleeding in the upper gastrointestinal tract. Barium studies revealed a gastric mass. At laparotomy, a large mass was found to involve the stomach, small intestine, and retroperitoneum. Biopsy revealed an anaplastic malignant tumor that could not be further classified. The patient failed to respond to further treatment with vinblastine plus vincristine or to other therapy and died. Autopsy revealed tumor involving the stomach, lungs, liver, duodenum, common bile duct, and retroperitoneal and mesenteric nodes. The histo-

[1] Received Sept 28, 1973; accepted Oct 25, 1973.

[2] Krebiozen®: CAS reg. No. 6020-87-7; creatine, monohydrate.

[3] Oncology Service, University of Southern California, Los Angeles.

[4] *Reprint requests to*: D.C. Stolinsky, MD, University of Southern California Oncology Service, 2825 S Hope St, Los Angeles, Calif 90007.

[5] Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate.

[6] Vinblastine: NSC-49842; CAS reg. No. 6449-03-2; vincaleukoblastine, sulfate (1:1), monohydrate.

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1).

logic appearance was characteristic of gastric carcinoma. Examination of the quadriceps muscles revealed the presence of cystic areas consistent with oil granulomas.

DISCUSSION

After extensive investigations, some lots of Krebiozen® were found to contain creatine and mineral oil, while other lots contained only mineral oil (1). On the basis of these investigations and detailed analysis of the case records of 504 patients who had received this material, Krebiozen® was determined to have no demonstrable efficacy against cancer and was banned from interstate shipment.

We are unaware of previous reports of recurrent masses at the injection sites, with temporal relation to upper respiratory tract infections. Mineral oil is known to cause granulomas (2), and one may speculate regarding a possible role in nonspecific immunologic stimulation. Of related interest is the observation that intraperitoneal injections of mineral oil in mice produce granulomas, hyperglobulinemia, and, eventually, plasma cell neoplasia (3). Unfortunately a biopsy was not performed during the recurrence of the lesions concurrent with a mild upper respiratory tract infection. The fact that the masses did not recur during the patient's final hospital stay, despite an episode of peritonitis, may be related to the immunosuppressive and anti-inflammatory effects of cytotoxic medication and of advanced cancer (4).

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Combination Chemotherapy With Adriamycin (NSC-123127) in Metastatic Mammary Carcinoma [1,2]

Cristina Brambilla, Mario De Lena, and Gianni Bonadonna [3,4]

Brief Reports and Preliminary Communications

In the past 4 years adriamycin has been found to exert a promising therapeutic effect on advanced carcinoma of the breast. A recent phase II evaluation (1) yielded complete plus partial responses in 16 of 50 patients (32%). A recent report on vincristine showed that this drug was effective in a total of 24% of patients (good risk, 32%; poor risk, 14%) with advanced mammary carcinoma (2). The present investigation was undertaken as a pilot study with the following purposes: (*a*) to assess the effectiveness of the combination of adriamycin and vincristine, and (*b*) to evaluate prednisone given in addition to this combination. Each treatment group comprised randomly selected patients.

PATIENTS AND METHODS

From October 1972 to February 1973, 42 patients with a histologically proven diagnosis of metastatic mammary carcinoma were entered into the study. Patients who were older than 74 years, had a performance status lower than 50, and had a life expectancy of less than 6 weeks were excluded from this trial. Tumor measurements included physical examination, skeletal survey, chest roentgenogram, and liver scan.

Adriamycin was administered on Day 1 of each cycle as a single intravenous (iv) dose of 75 mg/m² to 24 of 36 evaluable patients. In the remaining 12 patients, adriamycin was injected at a lower dose level (60 mg/m² to eight patients and 40 mg/m² to four patients) because patients were either older than 60 years or showed extensive osseous metastases. In the absence of severe hematosuppression, treatment was repeated at 3-week intervals. Vincristine was injected iv on Days 1 and 8 of each cycle at a dose of 1.4 mg/m². Prednisone was given at a dose level of 100 mg/m²/day for the first 5 consecutive days. Upon progression of disease, patients received a triple combination with cyclophosphamide (100 mg/m²/day from Days 1 to 14, orally), 5-FU (600 mg/m² on Days 1 and 8, iv), and methotrexate (40 mg/m² on Days 1 and 8, iv). This combination was also given to patients who were in remission or had static disease after eight cycles of treatment to avoid the risk of cardiomyopathy.

The characteristics of the patient groups are summarized in table 1. Five patients were not considered evaluable in the group treated with adriamycin plus vincristine (two were lost to followup after the first and second cycle respectively without evidence of partial response, and three lacked adequate parameters on retrospective evaluation). One patient in the group given adriamycin, vincristine, and prednisone was not considered evaluable (early death after one cycle). Both patient populations are fairly comparable in terms of prior treatment, menopausal status, and site of dominant lesion. Prior chemotherapy consisted of alkylating agents, antimetabolites, and CCNU administered either as single agents or in combination.

The various types of responses as well as the overall response rate by metastatic site were fairly similar in the two groups (table 2). Partial recalcification of bone metastases was observed in only four of 16 patients. The median duration of response (4.5 months in each group) and median time to initial response (4.5 vs 3.5 weeks) were also similar. Before the eighth cycle, three patients treated with adriamycin and vincristine and seven treated with adria-

[1] Received Nov 22, 1973; accepted Nov 27, 1973.

[2] Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are included in the List of Compounds at the end of the text.

[3] Istituto Nazionale Tumori, Milan, Italy.

[4] *Reprint requests to:* Dr. Gianni Bonadonna, Istituto Nazionale Tumori, Via Venezian 1, 20133 Milan, Italy.

TABLE 1.—Characteristics of patient groups

	Adriamycin + vincristine group	Adriamycin + vincristine + prednisone group
No. of patients entered	20	22
No. of patients evaluable*	15	21
Median age (yrs)	56 (32–74)	47 (33–72)
No. of pretreated patients	11 (73%)	13 (62%)
Endocrine ablation	40%	33%
Hormones	33%	24%
Chemotherapy	47%	43%
Menopausal status		
0–1 yrs postmenopausal	5 (33%)	9 (43%)
1–5 yrs postmenopausal	4 (27%)	5 (24%)
>5 yrs postmenopausal	6 (40%)	7 (33%)
Dominant lesion		
Soft tissue	10 (67%)	12 (57%)
Visceral	3 (20%)	4 (19%)
Bone	2 (13%)	5 (24%)

*To be evaluable patients must have received a minimum of 2 treatment cycles.

TABLE 2.—Comparison of response

Type and site of response	Adriamycin + vincristine group (15 patients)	Adriamycin + vincristine + prednisone group (21 patients)
Response type		
None	3	5
Objective improvement	2	4
Partial (≥50%)	9	10
Complete	1	2
Complete + partial	10 (66%)	12 (57%)
Total No. with response	12 (80%)	16 (76%)
Site of response		
Soft tissues	9/12* (75%)	15/20 (75%)
Lungs	3/3	3/5
Pleura	0/1	0/1
Liver	0/1	1/4
Bones†	2/5	2/11
Uterus	—	1/1

*No. of patients with response/No. evaluable.
†Partial recalcification.

mycin, vincristine, and prednisone were switched to 5-FU, methotrexate, and cyclophosphamide because of disease progression. After the eighth cycle this latter treatment was given to a total of 15 patients still in remission or with static disease (seven from the first group and eight from the second group). Therefore, the duration of response after adriamycin and vincristine with or without prednisone cannot be fully evaluated in this study. Table 3 correlates menopausal status and disease-free interval with response. While there was a tendency for a lower response in patients with a longer free interval, our data failed to show that the best response occurred in women who were less than 1 year postmenopausal.

Toxic manifestations and their percentages of occurrence were as follows: leukopenia 89%, thrombocytopenia 17%, alopecia 97%, stomatitis 27%, gastrointestinal disturbances 47%, and neurotoxicity 72%. Only three patients had a decrease in leukocyte count to less than 1500 cells/mm^3. One patient had a platelet

TABLE 3.—Menopausal status and disease-free interval in relation to response in both groups

	Disease-free interval		Menopausal status	
Years	No. of patients	No. of PR + CR	No. of patients	No. of PR + CR
0–1	19	13 (68%)	14	8 (57%)
1–5	10	6 (60%)	9	5 (55%)
>5	7	3 (43%)	13	9 (69%)

count less than 50,000 platelets/mm³. Myelosuppression required a dose attenuation schedule. Corticosteroids were discontinued in the presence of concomitant infection in five patients.

In the present series, patients received 81% of the projected dose of adriamycin, 84% of vincristine, and 90% of prednisone without a significant difference between the two groups regarding myelosuppressive drugs. The maximum total dose of adriamycin was 562 mg/m². In only four patients did the total dose of adriamycin exceed 500 mg/m².

COMMENT

The results of this preliminary trial with adriamycin and vincristine show that this combination produced complete plus partial remissions in more than 50% of patients with metastatic breast cancer. Despite the limited and selected series of patients, the response rate appears comparable to that achieved by the Eastern Cooperative Oncology Group with cyclophosphamide, 5-FU, and methotrexate,[5] and, more important, it appears comparable to that obtained with the quintuple Cooper's regimen or modifications of it (3). Furthermore, our data show that prednisone was of no value in this particular combination approach. Unfortunately, our study did not include a series of patients treated with adriamycin only. Therefore, we are not in the position to determine with certainty if comparable results could be obtained with adriamycin alone.

Collectively, the results of our combination appear promising for the treatment of metastatic breast cancer. The incidence of complete plus partial responses (even in previously treated patients), the prompt regression which appears peculiar of adriamycin, and the acceptable morbidity which allowed the administration of more than 80% of both adriamycin and vincristine should be taken into consideration for future studies. Since most of the current investigations seem to be based on different modifications of the Cooper's five-drug regimen, there is, in our opinion, a need to use more than one effective drug combination in treatment failures. One such combination could be adriamycin plus vincristine, provided the dose of adriamycin does not exceed 600 mg/m².

[5] Carbone PP. Personal communication.

LIST OF COMPOUNDS

Adriamycin: NSC-123127

CCNU: NSC-79037; CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan

5-FU: NSC-19893; CAS reg. No. 51-21-8; 5-fluorouracil

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]-methylamino]benzoyl]-

Prednisone: NSC-10023; CAS reg. No. 53-03-2; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-

Vincristine: NSC-67574; CAS reg. No. 6449-03-2; leurocristine, sulfate (1:1)

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A New Antitumor Antibiotic, Carminomycin (NSC-180024) [1,2]

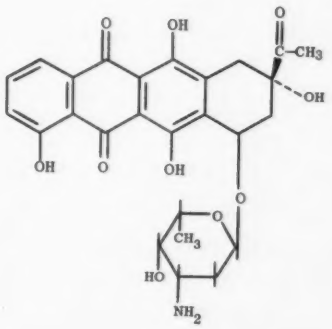
Georgij F. Gause, Maria G. Brazhnikova, and Vitalij A. Shorin [3]

Brief Reports and Preliminary Communications

In the course of screening for new antibiotics, a rare culture of actinomycete was isolated from soil which belonged to a new species, *Actinomadura carminata* (1). Mycelia of this culture contain the antibiotic carminomycin, which is formed on various nutrient media with organic nitrogen. Carminomycin was extracted from the mycelia by acetone at pH 4.0. The activity of this extract was assayed by the agar diffusion method using *Bacillus mycoides* as a test organism.

Carminomycin is produced by the culture of *A. carminata* as a complex consisting of five biologically active components: 1, 2, 3, 4, and 5. Carminomycins 2 and 3 are produced preferentially, constituting 50% and 30% of the carminomycin complex respectively. The other components are present in small amounts. The content of carminomycin 1 reaches 10%–12%, but under some conditions it may be completely absent. However, carminomycin 1 can be obtained from carminomycins 2 and 3 by means of mild acid hydrolysis of these variants. Carminomycin 1 is most interesting from the chemotherapeutic point of view. It was obtained in the form of crystalline hydrochloride soluble in water, and its physical and chemical properties (elementary analysis; ultraviolet, visible, infrared spectra and others) show that carminomycin 1 belongs to the anthracyclines. When carminomycin 1 is subjected to hydrolysis by 0.1 N hydrochloric acid at 100 C for 30 minutes it is split off into aminosugar and aglycone (carminomycinone) (2,3).

Study of the aminosugar has shown its identity to be daunosamine, which was isolated earlier from daunorubicin.[4] However, the aglycone carminomycinone represents a new anthracyclinone which has not been described before. Crystalline carminomycinone and its acetate and methyl ethers have been investigated. Nuclear magnetic resonance spectrometry has shown that carminomycinone contains five OH groups, three of which are in the anthraquinone chromophore at C_4, C_6, and C_{11}. Carminomycinone differs from daunomycinone by the absence of a methoxyl group at C_4, which is substituted by a hydroxyl group. A glycosidic bond was found at C_7. Differences in the melting points of methyl ethers of carminomycinone and daunomycinone, as well as in the optical rotation of aglycones and their derivatives, indicate stereochemical differences between carminomycinone and daunomycinone (3). The chemical structure of carminomycin is shown below:



The chemotherapeutic action of carminomycin was studied in mice with transplantable tumors. Mice with L1210 leukemia were treated with two intravenous (iv) injections of carminomycin (each equal to 0.5 of the LD50). In these studies carminomycin was found to be more effective than daunorubicin

[1] Received Nov 30, 1973; accepted Dec 20, 1973.

[2] Carminomycin: 5,12-naphthacenedione, 8-acetyl-10-[(3-amino-2,3,6-trideoxy-α-L-lyxo-hexopyranoxyl)oxy]-7,8,9,10-tetrahydro-1,6,8,11-tetrahydroxy-, (8*S-cis*)-; karminomitsin.

[3] Institute of New Antibiotics, USSR Academy of Medical Sciences, Moscow.

[4] Daunorubicin: NSC-83142; CAS reg. No. 23541-50-6.

used in doses of equivalent toxicity (4). Carminomycin inhibits the growth of lymphosarcoma in mice (strain Lyo-1) by 90% at 1.3 mg/kg. In these experiments the LD10 was 1.8 mg/kg.

Carminomycin at a dose of 0.4 of the LD50 injected iv three times with intervals of 120 hours inhibits growth of cancer of the lower part of the esophagus in mice (strain OZ–5) by 74%; at this dosage it inhibits the growth of bronchogenic cancer of the lung in mice (strain RL) by 72%. Carminomycin at an increased dosage of 0.5 of the LD50 inhibits growth of these tumors by 80% and 95% respectively.

The single intraperitoneal (ip), iv, subcutaneous (sc), and oral LD50s of carminomycin given to white mice are 1.3, 3.7, 3.8, and 7.3 mg/kg respectively. When compared to daunorubicin, carminomycin is four times more toxic when given ip, seven times more toxic when given iv, 11 times more toxic when given sc, and 27 times more toxic when given orally (5,6). Such strong differences in toxicity between carminomycin and daunorubicin given by the oral route are due to much better absorption of carminomycin from the gastrointestinal tract as compared to daunorubicin.

In studies with dogs it was observed that single and multiple iv injections of carminomycin, even in toxic and lethal doses, do not alter the functioning of the liver and kidney, do not alter the levels of sugar and electrolytes in the blood, and do not cause changes in electrocardiographic tests. The histopathologic study of organs in dogs killed after administration of carminomycin in toxic doses has not shown any irreversible toxic effects. Carminomycin possesses lymphotrophic action and affects lymphoid tissue in the spleen and lymphatic nodes. The death of dogs from the toxic doses of carminomycin results from aplasia of the bone marrow (6).

Irreversible inhibition of hematopoiesis appears after a single iv injection of the antibiotic at a dose of 1 mg/kg and also after ten daily iv injections of carminomycin each at a dose of 0.15 mg/kg. Severe but reversible inhibition of hematopoiesis in dogs was recorded when carminomycin was injected daily for 5 days at a dose of 0.15 mg/kg. The course can be repeated after an interval of 1 month. Similar severe reversible inhibition of hematopoiesis is observed after five daily oral administrations of carminomycin each at a dose of 0.30 mg/kg. If carminomycin is injected iv two times weekly in a single dose of 0.15 mg/kg for 5 weeks, alterations in the blood are only moderate.

The study of the pharmacokinetics of carminomycin in rabbits has shown that after iv injection at a dose of 1 mg/kg and after oral administration at a dose of 2 mg/kg the drug can be found in the blood for 3–5 hours (5). Carminomycin penetrates practically all tissues and organs and can be detected in the spleen, kidney, lungs, and liver for 3–5 hours after administration. However, the largest concentration is in the spleen, where it can be detected 24 hours after administration. Carminomycin is excreted with the bile (up to 11% of the injected dose) and with the urine (2.5%– 4.0%).

Iv injections of carminomycin given to cats narcotized by urethane in doses from 1 to 5 mg/kg produced no alterations in the blood pressure or in the frequency and amplitude of the respiratory movements. No alterations were noted on their cardiograms.

At present, the phase I study of carminomycin in the treatment of malignant tumors is completed and the results will be reported in a separate paper.

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Effects of Patient Selection on Results of Phase II Chemotherapy Trials in Gastrointestinal Cancer [1,2]

Charles G. Moertel, Allan J. Schutt, R. G. Hahn, and R. J. Reitemeier [3]

Brief Reports and Preliminary Communications

The basic treatment for patients with advanced gastrointestinal cancer is the phase II trial—a search for new tools of true therapeutic effectiveness. The only widely used chemotherapeutic agent for this most common group of malignant neoplasms is 5-fluorouracil (5-FU),[4] a drug that was developed some 15 years ago. Regardless of how this drug is given, it will produce only transient and usually incomplete tumor shrinkage for a small number of patients and there is no evidence that it adds to patient survival.

Our failure to identify any new agents that would be of significant value to the patient with gastrointestinal cancer can perhaps be rationalized by the presumption that no truly effective drugs have been placed in our hands. This may be so, but we must also admit the possibility that the methodology we have employed has not been adequate enough to meet this challenge.

There are countless factors that may favorably or unfavorably influence the results of a phase II trial, but perhaps the most important of these relates to patient selection. The purpose of this communication is to examine two facets of patient selection that may influence the evaluation of a new agent for the treatment of gastrointestinal cancer: prior therapy with cytotoxic drugs and performance status.

The Influence of Prior Therapy With Cytotoxic Drugs

Although there is no solid evidence that 5-FU therapy contributes anything of value to the overall group of patients treated, it has, nevertheless, been accepted by many as "standard" therapy for gastrointestinal cancer and has been marketed for this purpose. Whenever "standard" therapy is available for any given disease one must face the ethical problem of whether a patient should be exposed to a new and experimental regimen without first demonstrating his lack of response to that standard. The argument is raised, "Why not give the patient 5-FU first? After all, if it doesn't work the patient can always be put into a phase II study later." In a number of published phase II studies of new drugs used for the treatment of gastrointestinal cancer we find that essentially all patients entered have previously been exposed to 5-FU. It seems pertinent to examine the role of prior chemotherapy in the results of new drug evaluations.

In figure 1 we have plotted the survival of 484 consecutively admitted untreated patients measured from diagnosis of unresectable colorectal cancer in contrast to the survival of 289 patients measured after they have been declared to be 5-FU failures. (Patients are considered 5-FU failures if they do not respond to an adequate trial of 5-FU or if they initially respond and their disease progresses in spite of continued therapy.) The untreated patient survives a median time of almost 7 months, whereas the 5-FU failure survives just over 2 months. This latter group is therefore essentially in a preterminal condition. Overall, their performance level, nutritional status, liver function, and, quite possibly, immunocompetence have markedly deteriorated. It may be presumed that any or all of these factors could significantly influence the patients' responses to a new agent. Evidence for this presumption is shown in tables 1 and 2 where we have compared objective response rates between previously treated and untreated patients. These patients were studied in phase II trials that used inter-

[1] Received Dec 4, 1973; accepted Dec 7, 1973.

[2] Supported in part by Public Health Service grant CA-11911-01 from the National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3] Mayo Clinic and Mayo Foundation, Rochester, Minn.

[4] 5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU.

TABLE 1.—Therapeutic activity of the "phosphamides" in large bowel cancer: effect of previous cytotoxic drug exposure

Drug	Objective response * (>50%)	
	Prior treatment	No prior treatment
Cyclophosphamide	0/12 †	4/13 (31%)
Isophosphamide	0/16	2/16 (12%)

* Response was observed any time after the start of therapy.

† No. of patients responding/total No. of patients treated.

TABLE 2.—Therapeutic activity of the nitrosoureas in large bowel cancer: effect of previous cytotoxic drug exposure

Drug	Objective response * (>50%)	
	Prior treatment	No prior treatment
BCNU	4/42 † (9.5%)	4/22 (18%)
Methyl-CCNU	3/24 (12.5%)	4/16 (25%)

* Response was observed 8 weeks after the start of therapy.

† No. of patients responding/total No. of patients treated.

mittent high-dose cyclophosphamide and isophosphamide, and the nitrosoureas, BCNU and methyl-CCNU.[5] Each of these four agents produces a superior response rate among previously untreated patients.

The Influence of Performance Status

Frequently, treatment in a phase II study program is offered only to patients who have become seriously disabled from their malignant disease with the idea that the patient has really

[5] Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan.

Isophosphamide: NSC-109724; CAS reg. No. 3778-73-2; 1,3,2-oxazaphosphorine, 3-(2-chloroethyl)-2-[(2-chloroethyl)amino]tetrahydro-, 2-oxide; ifosphamide.

BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-.

Methyl-CCNU: NSC-95441; CAS reg. No. 33073-59-5; urea, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitroso-.

All drugs used in the current study were supplied by Cancer Therapy Evaluation, Division of Cancer Treatment, NCI.

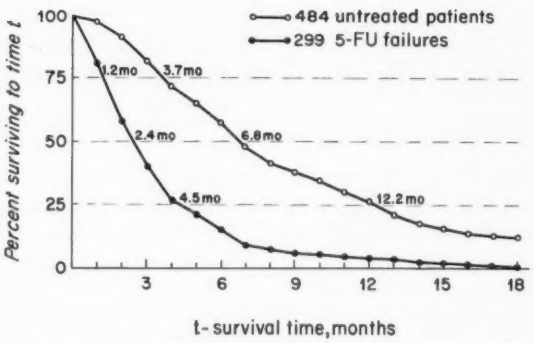

FIGURE 1.—Comparative survival between untreated colorectal cancer patients measured from diagnosis of unresectable malignant disease and treated colorectal cancer patients measured from the time they were deemed unresponsive to 5-FU therapy.

very little to lose should the drug prove inactive or cause a serious toxic reaction. To test the relationship between a patient's disability from malignant disease and his response to chemotherapeutic agents we have related performance status to our objective response rates in colorectal cancer patients treated with the fluorinated pyrimidines, 5-FU and 5-fluoro-2′-deoxyuridine.[6] Performance status was graded according to the Eastern Cooperative Oncology Group (ECOG) scale:

grade 0—fully active, able to carry on all pre-disease performance without restrictions;

grade 1—restricted in strenuous activity but ambulatory and able to carry out work of a light and sedentary nature, eg, light house work or office work;

grade 2—ambulatory and capable of all self-care but unable to carry out any work activity, up and about more than 50% of waking hours;

grade 3—capable of only limited self-care, confined to bed or chair more than 50% of waking hours;

grade 4—completely disabled, cannot carry on any self-care, and totally confined to bed or chair.

[6] 5-Fluoro-2′-deoxyuridine: NSC-27640; 5-FUDR.

This is in essence a modification of the Karnofsky scale except that the Karnofsky scale also considers symptoms which might not necessarily produce disability.

In figure 2 it is obvious that performance status had a striking and highly significant effect on objective response. Patients with a grade 0 (fully active) have a very respectable response rate of 31%. Those patients with a grade of 1 (only slightly impaired activity) have a significantly reduced response rate of 17%. Patients with a grade of 2 (unable to work but still ambulatory) drop significantly again to a 9% response rate. With a grade of 3 (confined 50% of the time) only a 7% response rate is seen; and although no grade 4 (totally disabled) patients were treated, it might be guessed that they would show essentially no response at all. Performance status is the single most sensitive guide to fluorinated pyrimidine response that we have encountered.

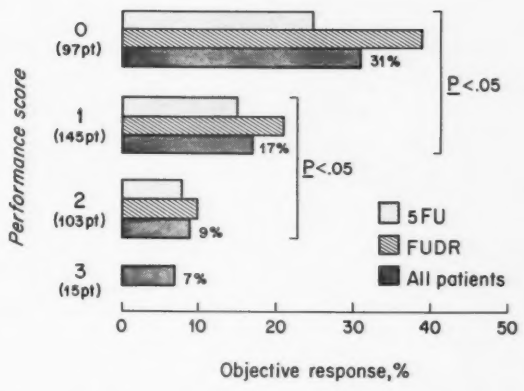

FIGURE 2.—Performance level (ECOG scale) and objective response to the fluorinated pyrimidines in colorectal cancer.

DISCUSSION

Current chemotherapy regimens for the treatment of patients with gastrointestinal cancer are grossly unsatisfactory. 5-FU alone has been tried by many routes and dosage schedules, and although this drug is of considerable historical interest, its therapeutic value is minimal at best. To insist on 5-FU as standard therapy for advanced gastrointestinal cancer offers precious little to today's patient and is a distinct disservice to tomorrow's patient.

If we are to make any progress in the treatment of this resistant group of neoplasms, it must be through the development of new treatment modalities. To test a new agent or approach in only the 5-FU failure or the patient who is seriously disabled and passing into the terminal stages of his disease, is to play a losing game. Negative results must be anticipated even with regimens of considerable therapeutic activity.

Recent Advances in the Management of Disseminated Sarcomas [1]

A Symposium Presented at the Ninth Annual Scientific Meeting of the American Society of Clinical Oncology

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Atlantic City, New Jersey

April 11, 1973

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[1] Received Oct 10, 1973; accepted Oct 25, 1973.

Editor's Introduction

Although sarcomas account for no more than 1% or 2% of all entries in most tumor registries, we, as oncologists, are frequently asked to advise in the management of patients with metastatic or inoperable sarcoma. Until recently, the outlook for such patients was as dismal as one could imagine. However, several recent advances have allowed for a certain degree of optimism for our current patients and even more for our future ones. The purely nihilistic approach to the patient with disseminated sarcoma can now be refuted with data.

The recent advances that I refer to include advances in basic science, especially immunology, and therapeutic advances by medical and surgical oncologists. A sampling of these therapeutic advances is contained in this Symposium.

Peter H. Wiernik

Combination Chemotherapy for Metastatic Sarcoma

Sarcoma Symposium

Jeffrey A. Gottlieb [1,2]

SUMMARY

Single drug studies with adriamycin in the Southwest Cancer Chemotherapy Study Group (SWCCSG) and elsewhere have revealed a response rate in soft tissue and bone sarcomas of approximately 25%–30%. Similar studies with 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (NSC-45388) have shown a response rate of approximately 15%. An SWCCSG protocol using combination chemotherapy with adriamycin and NSC-45388 has elicited 22 complete and 63 partial remissions in 200 evaluable patients treated, for an overall response rate of 42.5%. With 19 of 22 complete responders still in remission, the median duration of complete response is 12+ months. Responding cell types have included all the major different types of sarcoma with the exception of chondrosarcoma. Recently, a third drug, vincristine, has been added to the combination and the current response rate with this three-drug combination is 48% (19 of 40 patients). Adriamycin combinations appear to offer a treatment program of definite therapeutic value that improves both the frequency and the duration of response in patients with advanced sarcoma.

[Cancer Chemother Rep 58:265–270, 1974]

During 1970–1971, two broad phase II studies involving sarcomas, the first with 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (NSC-45388) (1) and the second with adriamycin (2), were carried out by the Southwest Cancer Chemotherapy Study Group (SWCCSG). These two agents proved to have definite activity in patients with a wide variety of soft tissue and bony sarcomas despite extensive previous therapy with surgery, radiotherapy, and the commonly used chemotherapeutic agents vincristine, actinomycin D, and cyclophosphamide, given alone or in combination.[3] Table 1 is a summary of the literature (including our own data) that existed when we first considered combining the two drugs. As the table indicates, adriamycin has about a 27% response rate and NSC-45388 has a lower one of about 15%. These data have since been augmented by a number of additional studies, and data on file at Cancer Therapy Evaluation, DCT, NCI indicate a continuing response rate of 25%–30% in approximately 250 patients with sarcomas treated with adriamycin.

In 1971, the Southern Research Institute began some studies with us which showed that

[1] Chemotherapy Service, Department of Developmental Therapeutics, M. D. Anderson Hospital and Tumor Institute, Houston, Tex.

[2] Supported in part by Public Health Service grants CA-05831, CA-10379, and CA-03754 from the National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3] NSC-45388: CAS reg. No. 4342-03-4; DIC.
Adriamycin: NSC-123127.
Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1).
Actinomycin D: NSC-3053.
Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate.
All drugs used in the current study were supplied by Cancer Therapy Evaluation, Division of Cancer Treatment (DCT), NCI.

TABLE 1.—Literature summary of sarcomas responding to adriamycin or NSC-45388*

Diagnosis	Adriamycin		NSC-45388	
	No. of patients	No. of remissions (complete/partial)	No. of patients	No. of remissions (complete/partial)
Sarcoma, unspecified and miscellaneous	37	—/5	7	—/—
Osteogenic sarcoma	35	2/8	6	—/1
Ewing's sarcoma	20	—/12	2	—/—
Rhabdomyosarcoma	16	—/2	13	—/2
Chondrosarcoma	8	—/1	1	—/—
Fibrosarcoma	5	—/3	11	—/1
Liposarcoma	4	—/2	2	—/—
Leiomyosarcoma	3	—/—	24	1/5
Synovial-cell sarcoma	3	1/2	1	—/—
Total	131	3/35 (29%)	67	1/9 (15%)

*From Gottlieb et al (ref 3).

when adriamycin and NSC-45388 were combined, essentially in full dosage, animals bearing a number of different solid tumors had an increase in lifespan compared with the lifespans seen with these drugs used singly (4). At the same time, we had started a small pilot study at M.D. Anderson Hospital in which we also observed that the two drugs could be given together with little increase in toxicity.

The regimen that we finally decided upon was adriamycin, 60 mg/m² given intravenously (iv) on Day 1 only, and NSC-45388, 250 mg/m² given iv on Days 1–5. These are essentially full doses of both drugs. Patients whose bone marrow reserves were deemed inadequate from their prior therapy received a 20% lower dosage (3). We recently added a third drug, vincristine, which is given at a dose of 1 mg/m² with a maximum dose of 1.5 mg on Day 1 and on Day 5; the entire regimen is repeated every 22 days (5).

We presented the initial report of the adriamycin–NSC-45388 combination at the annual meeting of the American Society of Clinical Oncology in 1972 (3).

At that time there were 100 patients analyzed and an overall response rate of 42% was elicited. Table 2 shows the current status of this two-drug study as well as the three-drug study which includes vincristine. A total of 200 evaluable patients were treated with adriamycin–NSC-45388 and 40 evaluable patients were treated with the three-drug program. As indicated in table 2, 34 patients receiving the two-drug combination and five patients receiving the three-drug combination were considered inevaluable, usually because of early death due to disease. There were also some patients that failed to complete more than a single course. When we analyzed this last group, we found that a disproportionate number were young teenagers who were taking themselves off this study or convincing their doctors to do so. I emphasize this because I think that teenagers, who form an important part of the sarcoma population, have difficulty (psychologically) staying on a rigid protocol. This should be kept in mind in the planning of prophylactic adjuvant chemotherapy studies that are currently being considered for the treatment of osteogenic sarcoma.

The response rate in the two-drug study, as shown in table 2, indicates 22 complete remissions in 200 evaluable patients for an 11% complete response rate, with 63 more patients in partial remission for a 42.5% overall response rate. In the ongoing study of the three-drug combination the overall response rate is up to 48.5%. This is clearly not a significant difference, and the additional patients in this

TABLE 2.—Adriamycin combinations in sarcoma

	Adriamycin and NSC-45388 (SWCCSG protocol No. 445)	Vincristine, adriamycin, and NSC-45388 (SWCCSG protocol No. 7210)
No. of patients entered	234	45
No. of patients inevaluable	34	5
Early death (<3 wks of treatment)	16	4
<1 course completed	18	1
No. of evaluable patients	200	40
No. of complete remissions	22	2
No. of partial remissions	63	17
All responses/ No. of patients entered	85/235 (36%)	19/45 (42%)
All responses/ No. of patients evaluable	85/200 (42.5%)	19/40 (48.5%)
No. of patients entered too early to analyze	20	79

latter study must be analyzed before we can see if there will be a difference.

We are frequently asked if the 42.5% overall response rate achieved with NSC-45388 added to adriamycin is better than the 25%–30% response rate which the literature supports as the best observed with adriamycin alone. Statistically analyzed, the answer is "yes," but I think the important difference is shown in table 3, which lists the durations of response. As indicated, the median duration of complete response has not yet been reached but will be in excess of 1 year with 19 of the 22 complete responders still in remission. This is particularly encouraging and considerably better than the durations shown with adriamycin alone (usually 4 months) (2). The median duration for all patients in remission is now up to nearly 7 months with 52% of these patients still in remission. These response durations and rates are better than those reported in the literature for adult patients with sarcomas receiving conventional agents, and it is especially encouraging since two thirds of our patients had extensive previous therapy before receiving this regimen.

As shown in table 4, all the major types of sarcomas were included in our study. The response rate for the various types of sarcomas is fairly consistent at approximately 40%–45% with some exceptions. For instance, chondrosarcoma has yet to respond to the drug combinations. The reason may be that the sparce blood supply of this tumor (6) does not permit the drug to reach the tumor in adequate concentrations. Similarly, the response rate for liposarcoma is somewhat less than the rates for other diagnoses. Although not statistically different, the response rates for women in the two- and three-drug studies were better than those for men. This was also true in our studies with adriamycin alone.

TABLE 3.—Duration of response to adriamycin and NSC-45388 in 85 responders

Remission	Median response in mos (range)	No. of patients still in remission/ total No. of patients
Complete	12+ (3+–23+)	19/22 (86%)
Partial	5+ (1–23+)	25/63 (40%)
All responders	6.5+ (1–23+)	44/85 (52%)

TABLE 4.—Complete and partial response rates in 240 evaluable patients*

Diagnosis	Adriamycin and NSC-45388 (SWCCSG protocol No. 445)	Vincristine, adriamycin, and NSC-45388 (SWCCSG protocol No. 7210)	Combined drug therapy
Angiosarcoma	3/8	0/1	3/9 (33%)
Chondrosarcoma	0/9	0/1	0/10 —
Ewing's sarcoma	1/2	—	1/2 (50%)
Fibrosarcoma	11/23	3/6	14/29 (48%)
Leiomyosarcoma	17/36	5/10	22/46 (48%)
Liposarcoma	5/16	2/4	7/20 (35%)
Mesothelioma	4/9	0/2	4/11 (36%)
Neurofibrosarcoma	10/22	—	10/22 (45%)
Osteogenic sarcoma	13/31	1/5	14/36 (39%)
Rhabdomyosarcoma	7/17	2/3	9/20 (45%)
Synovial-cell sarcoma	5/8	—	5/8 (63%)
Undifferentiated sarcoma	9/19	6/8	15/27 (56%)
Totals	85/200 (42%)	19/40 (48%)	104/240 (43.4%)

*No. of complete plus partial remissions/total No. of evaluable patients.

Table 5 analyzes the response rate by the site of the primary tumor. There does not seem to be a great difference, but the response rates for tumors originating in the gastrointestinal tract and in the pelvis and trunk were somewhat less than those for other primary locations. It is interesting that neither adriamycin nor NSC–45388 has been effective against gastrointestinal adenocarcinoma (2,7), and neither seems to be very effective with sarcomas that originate in the gastrointestinal tract. The response rate by the site of metastases is shown in table 6. The major sites respond with relatively little difference; the skin and subcutaneous metastases respond slightly better, and the central nervous system lesions respond most poorly. Fortunately, the central nervous system is a rare site for metastatic sarcoma. Neither adriamycin nor NSC-45388 achieves a significant concentration in the cerebrospinal fluid (8,9), possibly accounting for the poor response rate in metastases involving the brain.

Toxicity from the adriamycin–NSC-45388 regimen is summarized in table 7. Myelosuppression was built into the protocol and has been described in detail in our previous communications (3). Nausea and vomiting are worse during the first few days of therapy and are mainly caused by NSC-45388. Alopecia is predominantly due to adriamycin. The other side effects are relatively insignificant.

In conclusion then, we feel that adriamycin has changed the picture in what one can offer patients with metastatic sarcomas. We feel confident that the drug combinations reported here are further improving the response rates and the durations of response in patients with advanced sarcoma.

TABLE 5.—Response by site of primary tumor in 200 patients receiving adriamycin and NSC-45388

Primary site	Responses/No. of evaluable patients	
Head and neck	11/19	(58%)
Genitourinary and uterus	9/17	(53%)
Retroperitoneum	13/26	(50%)
Unknown and miscellaneous	17/35	(49%)
Limb	27/70	(39%)
Pelvis and trunk	5/19	(26%)
Gastrointestinal tract	3/14	(21%)
Total	85/200	(42.5%)

TABLE 6.—Response by site of metastases in 200 patients receiving adriamycin and NSC-45388

Metastatic site	Responses/No. of evaluable patients*	
Miscellaneous	3/5	(60%)
Nodes, skin, and subcutaneous lesions	16/29	(55%)
Lung	60/134	(45%)
Soft tissue	21/50	(42%)
Liver	13/34	(38%)
Bone	10/29	(34%)
Central nervous system	1/7	(14%)

*Many patients had more than one metastatic site.

TABLE 7.—Toxic effects

Toxic effect	Incidence (%)
Nausea and vomiting	100
Alopecia	100
Myelosuppression	86
Stomatitis	15
Electrocardiogram abnormalities	7
Neurotoxic effects	7
Fever	6
Other (hepatic, skin, phlebitis, conjunctivitis, eosinophilia)	5

DISCUSSION

Dr. Wiernik: I must comment before we go on that Dr. Gottlieb's data suggest very strongly that two and perhaps three drugs including adriamycin show improvement in the response rate in metastatic sarcoma when compared to adriamycin alone. However, because of the undesirable combined effects of vincristine (neurotoxicity) and NSC-45388 (gastrointestinal toxicity and often unexpected marrow suppression) when these drugs are added to adriamycin, we would prefer a randomized, controlled clinical trial that compares single drug therapy to combination chemotherapy. At the Baltimore Cancer Research Center we have seen about the same response rate with adriamycin alone as is being reported here with two or three drugs, so I think we are in definite need of a controlled study.

Dr. Gottlieb: One of the things that should perhaps be put in perspective is that our study was done by a group, and the data are actually from 24 hospitals. Trying to compare these data with data from single institutions is perhaps erroneous in that there are different types of control over the patients.

Nevertheless, I would be the first to argue that the backbone of our protocol is clearly adriamycin. Now that we have isolated some of the important prognostic factors which were not available to us before and which indicate how stratification might now be done, control studies can be carried out. We have analyzed retrospectively our study of adriamycin alone which was done almost simultaneously with our combination study, and the populations are nearly identical. The response rates for the combinations are statistically superior. However, I think the important point is the improved response durations, and there we are especially encouraged by the combinations.

Question from the floor: Were these patients resistant to previous simultaneous combination chemotherapy? In other words, had they received a double or triple combination previous to this combination?

Dr. Gottlieb: Unlike Dr. Wiernik's study with adriamycin alone in which few of his sarcoma patients had received prior therapy (10), about two thirds of our patients had received extensive prior treatment before receiving this combination. Forty percent had previously not responded to vincristine, actinomycin D, and cyclophosphamide alone or in combination, and another 30% had received intensive radiotherapy. Our current studies have a slightly less advanced population. Fortunately there appears to be little cross-resistance between the conventional agents and the drugs used in our regimen.

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Pharmacokinetics of Adriamycin (NSC-123127) in Patients With Sarcomas [1]

Sarcoma Symposium

Robert S. Benjamin [2]

SUMMARY

Pharmacokinetic studies of adriamycin performed on patients with sarcomas and other tumors have revealed a prolonged plasma half-life for the drug and its metabolites. The intermittent single high-dosage schedule based upon this pharmacokinetic data has been at least as successful as any other dosage schedule and has substantially reduced the incidence of mucositis, a major manifestation of drug toxicity. Because of low urinary excretion, renal insufficiency should *not* be expected to increase adriamycin toxicity when the drug is given in full doses. On the other hand, because of the importance of biliary excretion in the pharmacodynamics of adriamycin, major dosage reductions are necessary to prevent life-threatening toxic reactions in patients with severe liver disease.

[Cancer Chemother Rep 58:271–273, 1974]

A new interest in therapy for patients with disseminated sarcomas has come about since the introduction of adriamycin into clinical trials and its initial success reported against these tumors from a number of centers (1–5). In collaboration with Dr. Peter H. Wiernik, Dr. Nicholas R. Bachur, Mr. Charles E. Riggs, Jr., and I have studied the pharmacokinetics of this drug. In this study we obtained information which has allowed us to increase the drug's therapeutic index.

MATERIAL AND METHODS

Adriamycin and its metabolites were extracted (*a*) from plasma with an acid alcohol solution according to the methods of Benjamin, Riggs, and Bachur (6); (*b*) from urine with N-butanol according to the methods of Benjamin, Riggs, and Bachur (6); and (*c*) from cerebrospinal fluid by the isopropanol method of Benjamin, Wiernik, and Bachur (7). They were measured fluorometrically (excitation, 470 nm; fluorescence, 585 nm).

RESULTS AND DISCUSSION

In our initial studies (6) we have shown that adriamycin disappears from plasma quite slowly after a single intravenous (iv) injection, and that following an initial distribution phase, the half-life for adriamycin and its metabolites is 26.7 hours (fig 1). The half-life of native adriamycin is somewhat shorter, but it is still sufficiently long to assure prolonged plasma levels after a single iv dose (fig 1). There is extensive metabolism to several as yet unidentified metabolites of unknown activity (6).

Adriamycin is thought to be most active in the S phase although it has activity throughout the cell cycle (8,9). The long biologic half-life of adriamycin permits tumoricidal

[1] Adriamycin was supplied by Cancer Therapy Evaluation, Division of Cancer Treatment (DCT), National Cancer Institute (NCI).

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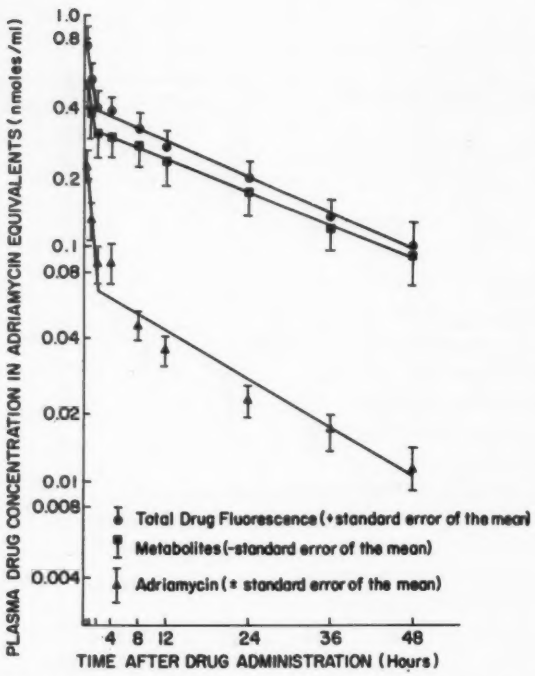

FIGURE 1.—Plasma disappearance curves of adriamycin and its metabolites. [Reprinted with permission from ref 6.]

levels of the drug to be present throughout the cell cycle after a single dose and might also permit toxic accumulation with repeated doses. An intermittent single high-dosage schedule would therefore seem logical. Although I do not wish to report here the details of our clinical trials which have been reported on earlier at this meeting (7,10), let me stress that the therapeutic efficacy which we have observed with a dosage schedule of 60 mg/m² every 3 weeks equals or surpasses that reported by others using different dosages and schedules (1–4).

In addition, toxic effects have been substantially reduced with our program. Mucositis is a serious and uncomfortable complication of chemotherapy that often leads to a life-threatening infection. The incidence of mucositis in studies using other dosage schedules increases in direct relation to the duration of the course of therapy. In early studies in which drug administration was continued until the appearance of toxic reactions, the incidence of mucositis approached 75% (1,3); for intermittent loading-dose schedules (usually daily administration for 3 days) with a rest period allowing for recovery from toxic reactions, the incidence of mucositis was about 40% (2); for our schedule, the incidence of mucositis was 9%, which is significantly lower (7).

A knowledge of the pharmacokinetics of adriamycin is useful in planning therapy for patients with impaired renal or hepatic function. Unlike daunorubicin,[3] a similar anthracycline antibiotic of which 25% of the administered dose is excreted into the urine in 5 days (11), less than 6% of the administered adriamycin is found in the urine after a similar period of time (6). In one patient with severe renal failure, we administered a full dose of adriamycin and found normal pharmacokinetics and only mild toxic reactions (7).

Adriamycin appears to be excreted primarily by the liver,[4] and in patients with severe liver disease (bilirubin >3 mg/100 ml or sulfobromophthalein [BSP] >15%) we have noted the plasma levels of this drug and its metabolites to be 4–5 times normal and the drug elimination to be delayed (fig 2). Toxic effects in such patients are extremely severe when full adriamycin doses are given, with three of eight patients (38%) having severe mucositis and all eight patients having severe pancytopenia which resulted in three drug-related deaths (7). We have subsequently reduced the dosage of adriamycin by 75% when severe liver disease occurs and by 50% when moderate liver disease (bilirubin 1.2–3 mg/100 ml or BSP 9%–15%) is present. By so doing, we have achieved plasma levels, toxic reactions, and response rates which approach those of patients with normal liver functions who are treated with full dosage (7). Although some patients with normal bilirubin but elevated BSP require dosage reduction, others do not. We therefore monitor plasma levels and toxic effects in these patients and escalate subsequent dosage to a normal level if possible. It should be noted also that bone marrow infil-

[3] Daunorubicin: NSC-83142; CAS reg. No. 23541-50-6; rubidomycin, hydrochloride; daunomycin (NSC-82151).

[4] Benjamin RS, Riggs CE, Jr, and Bachur NR. Unpublished data.

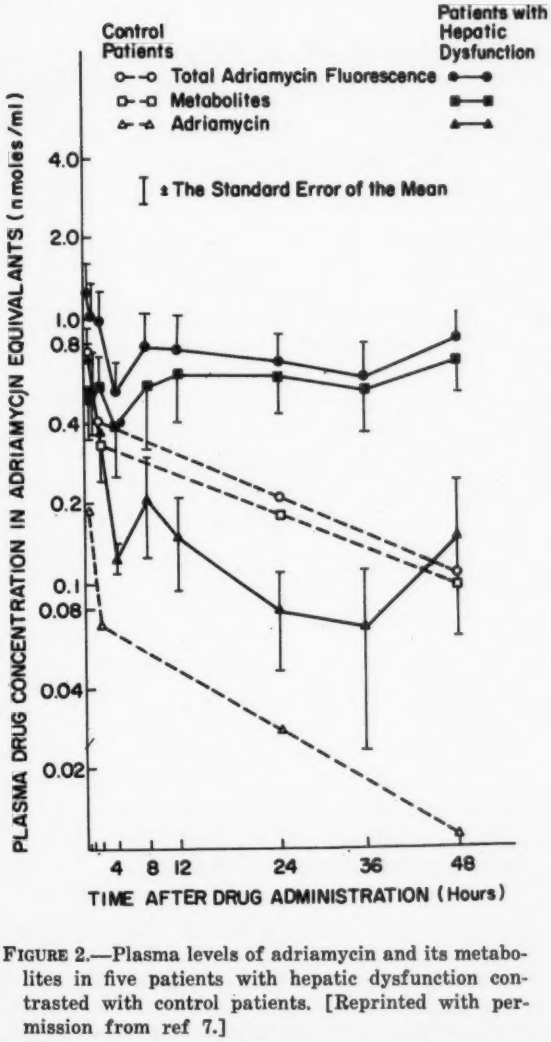

FIGURE 2.—Plasma levels of adriamycin and its metabolites in five patients with hepatic dysfunction contrasted with control patients. [Reprinted with permission from ref 7.]

tration with tumor cells and prior radiotherapy also increased the risk of hematologic toxicity from adriamycin in our study, but that prior chemotherapy did not appear to do so (7).

Although dosage reduction is mandatory in the presence of hyperbilirubinemia and is often essential in the presence of liver disease without hyperbilirubinemia, the resulting pharmacologically effective doses are normal. Because of bone marrow suppression from previous radiotherapy or tumor infiltration, dosage reduction results in lower *effective doses*. As a result, the margin of safety is lowered in such cases and a balance must be reached between acceptable toxic effects and desired therapeutic effects. The high therapeutic index offered by the intermittent single high-dosage schedule usually makes possible the administration of full doses to such patients provided that adequate supportive care is available to tide the patient over a brief period of severe bone marrow suppression (7).

Finally, we have been unable to detect adriamycin in cerebrospinal fluid after iv administration and have noted the disturbing appearance of cerebral metastases in two of our patients with sarcomas who continued to respond systemically to adriamycin (7). The presence of intracerebral disease appears, therefore, to be an obstacle to the successful use of adriamycin and is probably an indication for treatment with agents known to cross the blood-brain barrier.

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Progress Report on High-Dose Methotrexate (NSC-740) With Citrovorum Rescue in the Treatment of Metastatic Bone Tumors [1]

Norman Jaffe [2]

SUMMARY

High-dose methotrexate with citrovorum rescue will cause regression of metastatic osteogenic sarcomas in a minority of patients. At least four treatments (up to 250 mg/kg) should be given before the tumor is determined to be nonresponsive. In some cases of osteogenic sarcoma responses may be seen only after prolonged 24-hour infusions of methotrexate at high doses. Vincristine administered ½ hour prior to methotrexate infusion may enhance uptake of methotrexate by tumor cells.

[Cancer Chemother Rep 58:275–280, 1974]

The Children's Cancer Research Foundation commenced studies with high-dose methotrexate in metastatic osteogenic sarcoma in February 1971. To date, 14 patients have undergone treatment (table 1). Metastases in these patients were present in the lungs, soft tissue, or bone. Included in this series were two patients with metastatic osteochondrosarcoma (generally considered a more resistant tumor) and one patient with multifocal sclerosing osteogenic sarcoma involving several different parts of the skeleton. The duration of disease prior to high-dose methotrexate treatment ranged from 2 to 14 months.

Full details of the procedure for administering high-dose methotrexate with citrovorum rescue have been reported elsewhere (1) and are outlined in table 2. Briefly, methotrexate, dissolved in 250–500 ml of dextrose and water, is administered intravenously over a 6-hour period, followed by citrovorum factor injections 2 hours later. The initial dose of methotrexate comprises 50 mg/kg with increments introduced at two or three weekly intervals, provided excessive toxicity is not encountered. The dose of methotrexate at which eradication of the tumor occurs is designated the "response dose" and a 50-mg/kg increment above this dose and the prescribed interval in the schedule are adopted for maintenance treatments. Citrovorum factor is administered in doses of 6–15 mg at six hourly intervals. The exact dose is dependent upon the surface area of the patient and the amount of methotrexate administered.

If a response to chemotherapy had not been obtained after a suitable trial, or if complications appeared imminent during treatment, radiation therapy was administered.

A complete remission was defined as the disappearance of all measurable lesions. A partial remission was defined as a disappearance of tumor or a decrease in tumor size by 25% of one or more measurable lesions. The duration of response was measured from the time of disappearance or reduction in tumor to the time of tumor regrowth.

[1] Methotrexate: CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]-benzoyl]-. Supplied by Cancer Therapy Evaluation, Division of Cancer Treatment, National Cancer Institute.

[2] Outpatient Tumor Therapy Clinics, Children's Cancer Research Foundation, Boston, Mass.

TABLE 1.—Osteogenic sarcoma patients treated with high-dose methotrexate and citrovorum rescue

No. of patients	14*
Age (yrs)	7–23
Metastases	Lungs, soft tissue, bone
Duration of disease prior to treatment (mos)	2–14

*Includes two patients with osteochondrosarcoma and one patient with multifocal sclerosing osteogenic sarcoma.

RESULTS

With methotrexate alone a complete response was obtained in two patients; metastases were located in the lungs and the duration of response varied from 2 to 21+ months. A partial response was obtained in two patients. Their metastases were located in the lungs and bone and the duration of response varied from 1 to 2 months. There were no responses in ten patients.

With the addition of radiation therapy (2), further responses were obtained. A complete response occurred in one patient (No. 1) who relapsed after achieving a complete response with methotrexate previously. A partial response was obtained in four patients in whom metastases were present in the lungs, soft tissue, and bone. The duration of response was 1–3 months (table 3).

Responses obtained with methotrexate alone were distinct at the 200-mg/kg dose. In one of the two cases where a complete remission was achieved, the lesions initially appeared to increase in size. This was attributed to edema and was followed by gradual disappearance. The other complete response (patient No. 1) was complicated by bilateral pneumothoraces. With re-expansion of the lungs, complete disappearance of the metastases was noted.

Among the patients who failed to respond to methotrexate alone were the two with osteochondrosarcoma and the one with the multifocal sclerosing sarcoma. Three of the ten patients who did not respond did not survive long enough to receive a dose of 200 mg/kg of methotrexate. One of the patients who initially achieved a partial response with 200 mg/kg of methotrexate and then relapsed failed to achieve a further response with doses escalated to 800 mg/kg. There were no toxic effects. These experiences would suggest that the critical dose response is of the order of 200 mg/kg of methotrexate.

The initial responses to methotrexate all occurred with a 6-hour "pulse" infusion. In three

TABLE 2.—Drug dosage and schedule

Interval between doses	Dose of methotrexate (mg/kg)	Citrovorum factor (mg)		
		≤ 0.5 m²	0.5–1.0 m²	≥ 1.0 m²
2 weeks	50, 100, 200	6	9	12
3 weeks	300, 400, 500	9	12	15

TABLE 3.—Responses to high-dose methotrexate and citrovorum rescue in patients with osteogenic sarcoma

Treatment	Type of response	No. of patients	Site	Duration of response (mos)
Methotrexate	Complete	2	Lungs	2 and 21+
Methotrexate	Partial	2	Lungs, bone	1 and 2
Methotrexate	None	10	Lungs, bone	—
Methotrexate plus radiation	Complete	1	Lungs	2
Methotrexate plus radiation	Partial	4	Lungs, soft tissue, bone	1–3

patients who relapsed, attempts were made to surmount resistance to methotrexate by prolonging the period of administration from 6 to 24 hours. One sixth to one tenth of the next prescribed dose in the 6-hour schedule of methotrexate was infused in 250 ml of 5% dextrose and water over 1 hour, followed by the remainder in 1000–1500 ml over 23 hours. The schedule of citrovorum factor was extended to 20 injections. One of the three patients achieved a partial response. This patient (No. 1) had previously achieved a complete response to high-dose methotrexate with citrovorum rescue but defaulted in maintenance treatments. Recurrence of the tumor was noted 2 months later (fig 1*a*). Six-hour infusions of methotrexate failed to produce a second response, but regression of the tumor was again achieved with radiation therapy (fig 1*b*). Maintenance treatment with 6-hour infusions of methotrexate was reinstituted, but recurrence of the tumor again occurred 2 months later (fig 2*a*). The 24-hour infusion of methotrexate was then administered, resulting in cavitation of several metastases (fig 2*b*). Full details of this patient's course and responses to treatment are outlined in figure 3.

Toxic Effects

Toxic effects with high-dose methotrexate treatments were unpredictable (table 4). The complete spectrum was not always reproducible in any one patient: a number of patients tolerated initial treatments poorly and yet experienced little difficulty with the higher administrations. Of a total of 120 treatments, stomatitis occurred in 24, vomiting in 20, skin eruptions in six, and renal aberrations in four. Approximately 15% of the treatments were associated with a depression in the hemogram. There were two possible drug-related deaths. Among the original 14 patients with metastases who entered into the study, only one remains alive and well and free of disease.

The limited measure of success achieved with high-dose methotrexate treatment in osteogenic sarcoma prompted an investigation of its effectiveness in other bone tumors. There were no responses in two patients with Ewing's sarcoma, but there was a dramatic partial re-

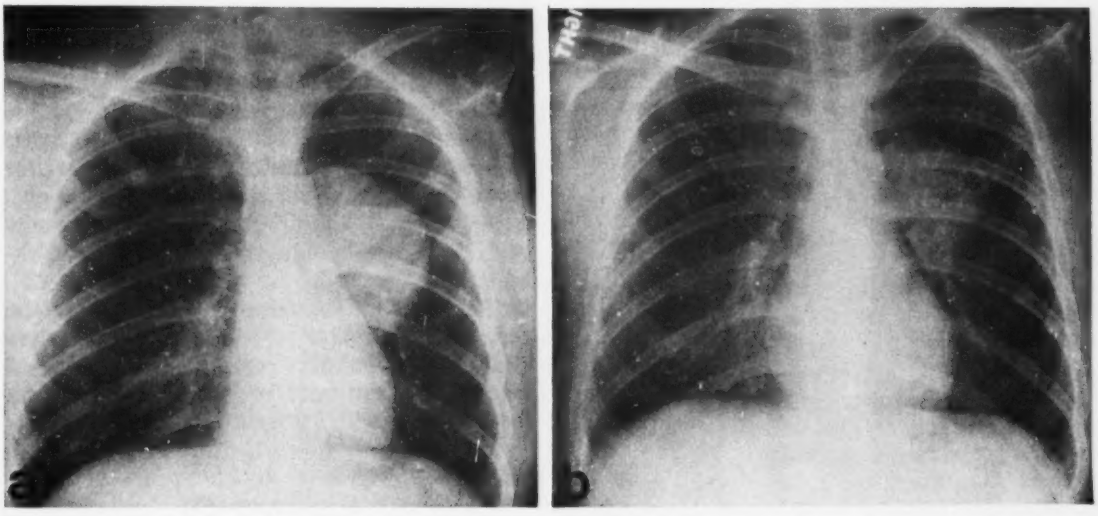

FIGURE 1.—*a*. Chest roentgenogram in patient No. 1 with osteogenic sarcoma who relapsed after achieving a complete response with "pulse" high-dose methotrexate (200 mg/kg) and citrovorum rescue. A small nodule in the right upper lobe and a large left hilar mediastinal mass are present. *b*. With radiation therapy, complete disappearance of the nodule in the right upper lobe and the mass in the left hilar area has occurred. The latter has been replaced by residual fibrosis.

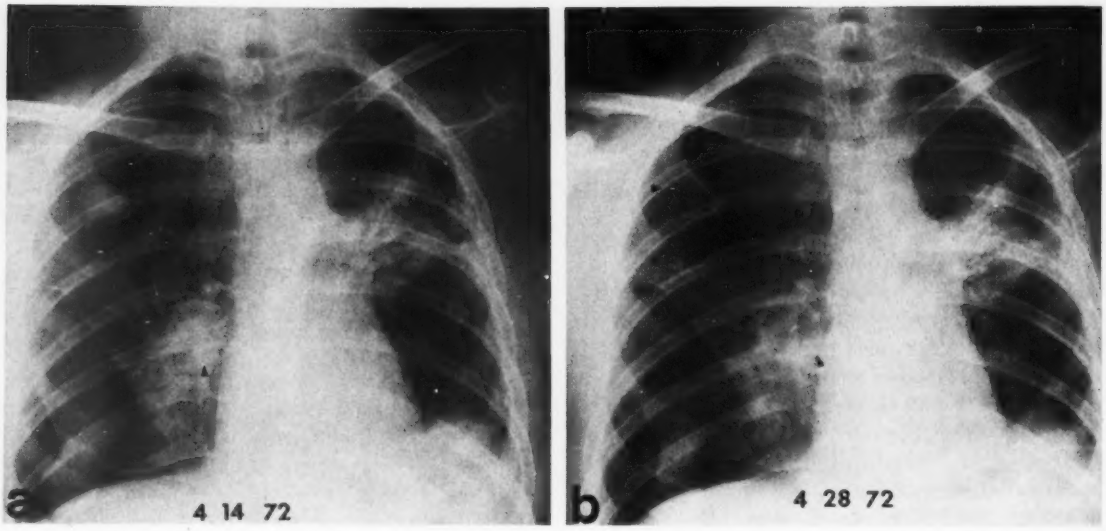

FIGURE 2.—*a*. Recurrence of tumor in patient No. 1 while receiving maintenance treatment with 6-hour "pulse" doses of high-dose methotrexate. Three nodules are present in the right hemithorax: two within the pulmonary parenchyma and one at the base of a small pneumothorax. Residual fibrosis is present in the left hilar area and a small pleural effusion is present at the left base. *b*. Cavitation of two of the pulmonary lesions in the right hemithorax has occurred following a 24-hour infusion of methotrexate. There has been no change in the metastatic nodule at the base of the right pneumothorax.

TABLE 4.—Toxic effects noted in 120 courses of high-dose methotrexate followed by citrovorum rescue

Toxic effect	No. of patients
Stomatitis	24
Vomiting	20
Skin eruptions	6
Renal aberrations	4
Bone marrow suppression	18

sponse in one patient (patient No. 2) with hemangioendotheliosarcoma (figs 4*a* and 4*b*). The response, which lasted for 2 months, occurred when 200 mg/kg of methotrexate was given. High-dose methotrexate was not continued according to the schedule at the request of the patient's parents.

Since the prognosis in osteogenic sarcoma is extremely poor, particularly once metastases develop, high-dose methotrexate treatment with citrovorum rescue is currently being administered as adjuvant treatment to all patients with this condition once the diagnosis is established. There may be a temporary interruption of treatment during radiation therapy to the primary lesion or during surgical ablation, but treatment is continued once these procedures are completed. The dose of methotrexate is escalated at intervals according to the schedule up to 250 mg/kg, which past experience would indicate is the critical dose for response. A 2-year course of treatment is proposed. Further, since preliminary evidence suggests that the uptake of methotrexate is enhanced by pretreatment with vincristine[3] (3), the latter is administered ½ hour before the commencement of the 6-hour infusion. Five patients are currently receiving this form of treatment. They have been free of metastases for periods ranging from 2 to 12 months.

QUESTIONS

Question from the floor: Can you be sure there is no residual tumor in areas of pulmonary fibrosis after treatment?

Dr. Jaffe: There was no recurrence of tumor in the

[3] Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1).

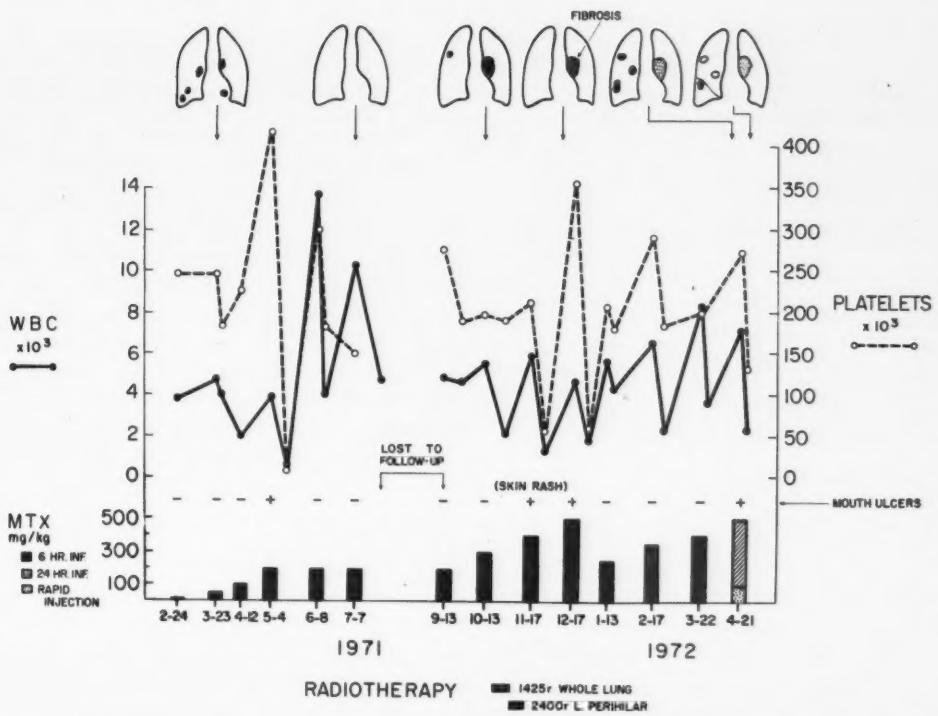

FIGURE 3.—Schematic diagram of lesions and response to treatment in patient No. 1.

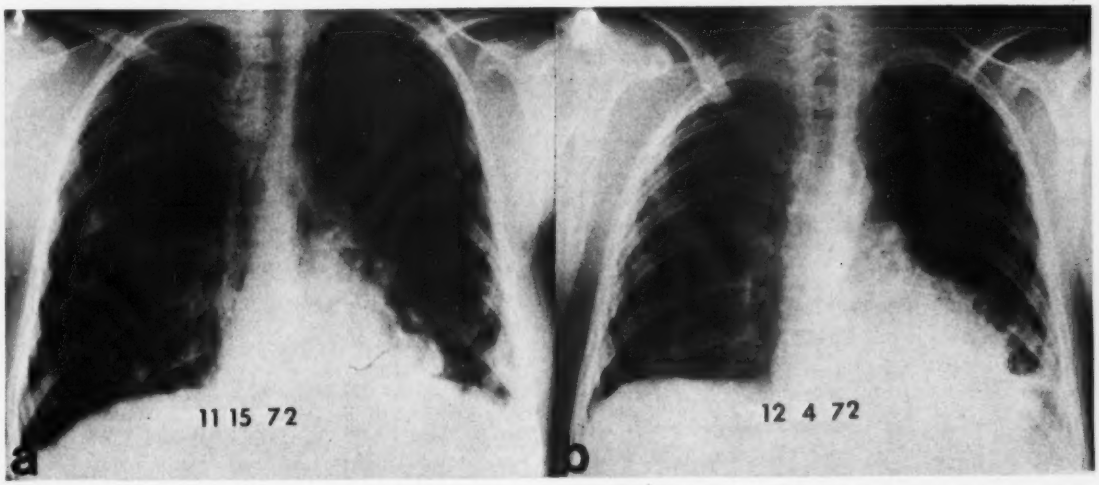

FIGURE 4.—*a*. Chest roentgenogram in patient No. 2 with hemangioendotheliosarcoma of the femur demonstrating bilateral pulmonary metastases. *b*. Disappearance of a large portion of the pulmonary metastases, particularly in the right lung following 200 mg/kg of methotrexate.

areas of fibrosis that we observed in any of the patients. Autopsy studies have failed to reveal identifiable tumor cells in areas of pulmonary fibrosis also.

Question from the floor: What is the average duration of treatment before maximal response is achieved?

Dr. Jaffe: Approximately 2½ months.

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Combination Chemotherapy for Embryonal Rhabdomyosarcoma [1]

Sarcoma Symposium

Jordan R. Wilbur [2]

SUMMARY

Combination chemotherapy with vincristine, actinomycin D, and cyclophosphamide has been utilized successfully in conjunction with radiation therapy and surgery in the treatment of rhabdomyosarcoma in children. This therapy is quite toxic and should be administered with caution. Other soft tissue sarcomas in children and adults have also been responsive to this type of therapy. The toxic effects of this treatment in adults may be more severe than those which occur in children.

[Cancer Chemother Rep 58:281–284, 1974]

During the past several years, more intensive treatment of patients with rhabdomyosarcoma has resulted in marked improvements in long-term survival (1–3).[3] The combination of vincristine, actinomycin D, and cyclophosphamide (VAC),[4] usually given concurrently with radiation therapy, has been the most successful treatment program for rhabdomyosarcoma. This drug combination has been commonly referred to as VAC chemotherapy, and is usually given with the doses and timing indicated in table 1. More intensive therapy may be indicated for more extensive tumor (table 2). Three aspects of this therapy program will be discussed: (*a*) the sequencing of the therapy in relation to the volume and location of the tumor; (*b*) the results of this treatment program; and (*c*) the prevention and treatment of toxic effects which may occur.

The primary goal of this therapeutic program is the eradication of both localized bulk tumor and occult metastatic disease. Surgical removal of bulk tumor remains the preferred treatment when the lesion is located in a site where the surgery will not cause significant residual defect or jeopardize the patient's life (course A, table 3). In some situations either chemotherapy or radiation therapy or both may be given prior to surgery to shrink the bulky tumor to make surgery feasible or less extensive (courses B and C, table 3).

Radiation therapy is used to eradicate residual bulk tumor or residual microscopic tumor or to shrink bulk tumor to improve operability. The dose of radiation therapy used should be the maximum possible within the limits of tolerance for the surrounding normal tissue. Particular regard must be given to the age of the patient and the long-term function and cosmetic effect of the radiation on normal growing tissue. Consideration must always be given to the enhancement effect of actinomycin D on

[1] Supported in part by Public Health Service grant CA-03713 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare; and the Russell G. Adderley Research Fund.

[2] Children's Oncology Program, Children's Hospital at Stanford, Palo Alto, Calif.

[3] Donaldson S, Castro JR, Wilbur JR, et al. Rhabdomyosarcoma of head and neck in children: combination treatment by surgery, irradiation and chemotherapy. (Abstr) Presented at the American Radium Society meeting, Boca Raton, Fla, May 14, 1972.

[4] Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1).

Actinomycin D: NSC-3053.

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate.

radiation of normal tissue, as well as tumor tissue.

The purpose of the chemotherapy varies with the clinical location and the extent of the tumor. When the primary tumor has been surgically removed, the major purpose of chemotherapy is the eradication of occult distant metastases as well as the treatment of local microscopic residual tumor. With residual inoperable tumor, the goal may be either to shrink the tumor to achieve operability or to combine effects with radiation therapy to eradicate the tumor without surgery.

When gross metastatic tumor is present at the initiation of the therapy, more intensive combination chemotherapy will, in some instances, eradicate all of the tumor without surgery or radiation therapy. However, when the latter modalities of therapy can also be utilized, they will increase the possibility of long-term complete control of the tumor. The simultaneous utilization of radiation therapy and combination chemotherapy seems to have contributed to the increased percentage of patients with inoperable tumors who have achieved long-term control (4).

TABLE 1.—VAC (standard) schedule

	No. of doses		
Week	Vincristine (2 mg/m²/wk iv)*	Actinomycin D (0.75 mg/kg/course iv)†	Cyclophosphamide (2.5 mg/kg/day orally)
1	×	×××××	×××××××
2	×		×××××××
3	×		×××××××
4	×		×××××××
5	×		×××××××
6	×		×××××××
7	×		×××××××
8	×		×××××××
9	×		×××××××
10	×		×××××××
11	×		×××××××
12	×	×××××	×××××××

*Maximum single dose, 2 mg.

†Maximum daily dose, 0.5 mg. Total of five doses given over 5–8 days.

TABLE 2.—VAC pulse dose schedule

	No. of doses		
Week	Vincristine (2 mg/m²/dose iv)*	Actinomycin D (0.075 mg/kg/course iv)†	Cyclophosphamide (10 mg/kg/dose orally)‡
1	×	×××××	×××××××
2	×		
3			
4			
5			
6			
7	×	×××××	×××××××
8	×		
9			
10			
11	×		
12	×	×××××	×××××××

*Maximum single dose, 2 mg.

†Maximum daily dose, 0.5 mg. Total of five doses given over 5 days.

‡Total of seven doses given over 7 days.

TABLE 3.—Combined modality therapy

Course	Treatment	Week 0	1	2	3	4	5	6	7	8	9	10	11	12
A	Surgery	×												
	Radiotherapy		×	×	×	×	×	×						
	Chemotherapy		×	×	×	×	×	×	×	×	×	×	×	×
B	Surgery					×								
	Radiotherapy		×	×	×		×	×	×	×	×	×		
	Chemotherapy		×	×	×		×	×	×	×	×	×	×	×
C	Surgery							×						
	Radiotherapy								×	×	×	×	×	×
	Chemotherapy		×	×	×	×	×	×	×	×	×	×	×	×

RESULTS

In association with my colleagues, Drs. W. W. Sutow and M. P. Sullivan, the results of the initial treatment of a group of patients at the University of Texas M. D. Anderson Hospital between January 1967 and May 1971 have been reported in part elsewhere (1–3).[5] An updated followup as of March 1973 indicates that the group of patients previously evaluated as doing well with no evidence of disease are all continuing to do well.

Of the initial group of 32 patients with embryonal rhabdomyosarcoma who had not received previous chemotherapy or radiotherapy, 22 are alive and well with no evidence of disease since their initial treatment. Further study of this group reveals that all eight patients with operable disease are alive without evidence of disease recurrence and have had a median survival of 47 months.

Of the group with operable disease, ten of 16 are alive and well. Of the eight patients who had distant metastases, four are alive without disease. The median survival in the latter two groups is 47 months. In all patients chemotherapy was completed by 24 months. Thus, the median survival time after chemotherapy is almost 2 years. So far, no patients have had relapses after completing chemotherapy.

CASE REPORTS

Patient No. 1: Inoperable Disease

A 15-year-old girl had embryonal rhabdomyosarcoma involving the nasopharynx and oropharynx with probable node metastases to the neck. A tracheotomy was required due to respiratory obstruction secondary to the rapidly growing tumor. She had partial surgical removal of the portion of the tumor in the oropharynx. The patient received cobalt 60 irradiation (6000 rads in 6 weeks), with simultaneous actinomycin D and vincristine. Cyclophosphamide was given after completion of radiation therapy. Intensive supportive therapy was required for severe mucositis secondary to the combined effect of radiation and actinomycin D (subsequent actinomycin D was withheld due to the severity of the mucositis). A nasogastric feeding tube and 3 months of hospitalization were required.

All chemotherapy (daily oral cyclophosphamide) was completed at 2 years. A followup at 48 months showed that the patient is alive and well with no evidence of disease and is attending college. The residual complications are primarily recurrent sinusitis and postnasal drip. (This patient is one of the ten successes reported with inoperable disease.)

Patient No. 2: Metastatic Disease

A 2-year-old boy with embryonal rhabdomyosarcoma of the right face metastatic to all bones, lungs, and bone marrow and with multiple vertebral collapse received VAC chemotherapy. The results were pain relief within days, clearing of the bone marrow within 4 weeks, and over a 90% reduction in the face mass. Eight weeks after therapy was given he was in good condition with no gross evidence of residual disease except for a 1-cm area of

[5] *See* footnote 3.

residual tumor at the primary site. At 12 weeks all of his bones had completely healed and they appeared normal with no evidence of disease. Shortly thereafter, he became acutely ill and suddenly died secondary to previously undetected brain metastases.

Postmortem examination revealed no evidence of residual tumor in any sites except the brain and a small area of tumor in the primary site of the face. (This patient is one of the four failures mentioned of the eight patients reported with distant metastases.)

TOXICITY

The extent of toxicity of VAC chemotherapy depends on the intensity of the doses administered, the extent and location of the radiation field, and the age of the patient.

The most significant toxic effect is the profound neutropenia that occurs with the simultaneous administration of radiation therapy and chemotherapy. The larger the radiation field is, with its associated increased depletion effect on bone marrow reserve, the more prolonged and severe is the leukopenia. Thrombocytopenia may also be significant, but may readily be treated with a platelet transfusion. The intensity of the chemotherapy is also significant in its effect on the peripheral blood counts. When more intensive chemotherapy is required to reduce the tumor bulk or to treat known metastatic disease (pulse VAC therapy, *see* table 2), the granulocytopenia is most profound.

Pulse VAC therapy is very effective in the treatment of a variety of soft tissue sarcomas but it is very toxic. Around the tenth day after the initiation of therapy, the granulocyte count will drop to zero and remain at that level from 4 to 7 days, and occasionally longer. In adults receiving this therapy, bone marrow function has taken longer to recover than that of younger children. The risk of sepsis is high during this period, and suspected infections must be cultured and treated vigorously with antibiotics without waiting for culture results. The availability of granulocyte transfusion appears to help reduce the risk of this period of agranulocytosis.

Individual drug toxicity related to each agent may also occur. With simultaneous actinomycin D local radiation toxicity to lung, intestine, bladder, skin, and oral mucosa may be markedly intensified. The neurotoxic effects of vincristine include peripheral neuropathies and severe constipation (severe adynamic ileus with associated obstruction secondary to inspissated stool). This condition may occur more frequently during the 12 weekly doses of vincristine than during a shorter course. These toxic reactions occur more frequently and severely in adults and teenagers. When necessary, prophylactic measures with stool softeners, laxatives, and enemas will help to minimize this complication. Cyclophosphamide-induced hemorrhagic cystitis is a frequent problem with prolonged and intensive use of the drug. It will almost certainly occur in patients who also receive radiation to the bladder. Administration of large volumes of fluid orally or intravenously before, during, and after administration of cyclophosphamide can help to minimize the possibility of cystitis. This is of particular importance with pulse VAC therapy. Frequent emptying of the bladder is also of value in helping to minimize this complication. Nausea and vomiting can be severe, and strong antiemetic therapy is often required.

Reduction in the doses of therapy administered is often necessary because of severe toxic reactions or the gradual decrease in bone marrow reserve following multiple courses of therapy.

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Criteria for Resection of Sarcoma Metastatic to the Lung

Sarcoma Symposium

William L. Joseph [1]

SUMMARY

Tumor doubling time appears to be an accurate method for quantitation of tumor growth and an important criterion for evaluation of operability. This study suggests a more aggressive approach should be used in patients who have metastatic pulmonary disease with a prolonged doubling time, regardless of the cell type of the primary malignancy.

[Cancer Chemother Rep 58:285–290, 1974]

Surgical extirpation of isolated pulmonary metastatic lesions is indicated for a variety of reasons: (*a*) as a method of diagnosis, (*b*) for palliation, (*c*) for reduction of the antigenic tumor prior to immunotherapy, and (*d*) for cure. Prolonged survival and apparent cure of selected patients following surgical removal of metastatic neoplasms of the lung are well established. However, criteria for evaluating operability in these individuals have been arbitrary and vary among institutions and clinicians. As a result, a more uniform and precise method is needed for comparison of cases, evaluation of operability, and prediction of survival. The present report outlines my experience in utilizing tumor doubling time (TDT) as a method for the quantitation of the rate and pattern of tumor growth in a group of patients treated for malignant disease in whom lung metastases later developed without clinical evidence of extrapulmonary foci.

CLINICAL MATERIAL AND METHODS

Over a 10-year period, TDTs were plotted for 113 patients with metastatic pulmonary disease. There were 61 men and 52 women whose ages ranged from 10 to 83 years. Forty-seven of these individuals had lung metastases from carcinoma and two from seminoma, while the remaining 64 patients had pulmonary lesions following treatment of a primary sarcoma. When the pulmonary neoplasms first appeared on the chest roentgenograms, the primary malignancies for all patients included in this study showed no evidence of local recurrence and there were no tumor foci outside the lung. Except for patients treated by resection, no other form of therapy for the metastatic disease was instituted.

TDT was calculated according to the method of Collins et al (1). The growth rate of a tumor (expressed as TDT) can be calculated from changes in tumor volume over a known time interval. When the serially measured diameters of metastatic foci are plotted on semilogarithmic paper, a straight line is produced; the slope of this line represents the tumor growth rate (fig 1). The diameter of each metastatic nodule measured on a standard 72-inch target-film distance posteroanterior chest roentgenogram is plotted along the vertical axis against the time between determinations on the horizontal axis. A line is then drawn to connect all points that are plotted. Where this line crosses any two doubling lines, the horizontal distance in between represents the TDT in days. Many serial measurements are made over an extended period of time for every lesion.

[1] Department of Surgery, George Washington University Medical Center, Washington, DC.

RESULTS

The overall results of this study have been reported elsewhere (2). We found that with the possible exception of Ewing's sarcoma, there was considerable variation in TDT for each tumor cell type. Table 1 illustrates the growth rates of pulmonary metastases in the 64 patients with metastatic sarcoma. Since no histologic type of lesion was characterized by a particular TDT, these lesions, regardless of histologic type, were reclassified into three groups on the basis of growth rate as measured by the TDT. Out of the total series, 71 patients (rapid group) had a TDT of 20 days or less. For these patients the mean interval between primary resection and the onset of a metastatic nodule was 4.8 months. Seventeen patients (intermediate group) had a TDT of 21–40 days. In this group the mean interval was 11.1 months after resection of the original malignancy and the appearance of pulmonary tumor foci. The remaining 25 patients (slow group) had a TDT greater than 40 days. A lung lesion occurred in this group 1–120 months after operation (mean, 29.5 months).

Eighty-nine patients had no further treatment after the onset of metastatic lung lesions. The remaining 24 individuals had received some type of direct surgical excision of the metastatic tumor. The cumulative survival of both groups of patients was plotted by means of the actuarial life table method (figs 2 and 3). In the group of 64 untreated patients who had a TDT of less than 20 days, 63% were alive at 6 months while only 11% survived for over 1 year from the onset of lung metastases (fig 2). Eleven patients in the untreated group had a TDT of 21–40 days. Ninety percent were

TABLE 1.—Growth rates of pulmonary metastases in 64 patients with primary sarcoma

Cell type	No. of patients	Doubling time (days)
Ewing's sarcoma	8	7–18
Fibrosarcoma	12	13–340
Liposarcoma	6	11–100
Mesenchymoma	2	5–43
Osteogenic sarcoma	25	11–360
Rhabdomyosarcoma	5	10–20
Botryoides	2	16–19
Synovial-cell sarcoma	4	16–80

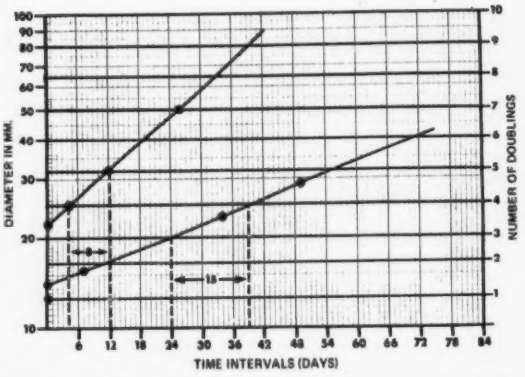

FIGURE 1.—Method of plotting TDT based on the direct measurement of the changing diameters of metastatic pulmonary nodules.

alive at 6 months; 45% survived for over 1 year. In both groups, there were no patients alive for over 18 months. There were seven patients treated by resection whose TDT was less than 20 days. Eighty-six percent were alive at 6 months while 28% survived for over 1 year after resection of a pulmonary disease. All six patients with a TDT of 21–40 days were alive at 6 months. Eighty-two percent in this group were alive at 1 year but none survived beyond 2 years.

There were 14 untreated patients with a TDT greater than 40 days (fig 3). All the patients in this group survived for at least 9 months after the onset of lung tumor foci. Eighty-six percent were alive at 1 year but none survived up to 2 years. Eleven patients

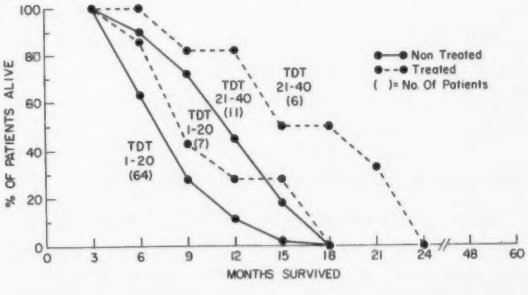

FIGURE 2.—Cumulative survival following onset of pulmonary metastasis in patients with TDT < 40 days.

who had received curative resection had a TDT of 40 days or longer. These patients have been followed from 5 to 62 months since resection of their metastatic foci. All of these patients are alive and free of disease at this time. In this particular group thoracotomy was either by wedge resection or by lobectomy (done when the disease was limited to one hemithorax). One patient required pneumonectomy because of the extent of the metastatic disease. Four patients had staged bilateral thoracotomies for their disease, while one patient had a simultaneous bilateral thoracotomy. In almost all cases, the metastatic disease was usually in a subpleural location. Often at the time of resection, additional subpleural foci were found which were not radiographically apparent. There were no operative deaths. There are too few patients in the operative groups to allow a definitive analysis of the results of operative intervention in those patients with a TDT of 40 days or less. However, it is obvious that the survival of patients who had resection and a TDT greater than 40 days is significantly greater, than that of the untreated patients with a TDT greater than 40 days and the two other operative groups with a shorter TDT. Six of the 11 patients who had resection and a TDT greater than 40 days had a sarcoma as the primary malignancy (table 2). The majority of these long-term survivors had multiple nodules either in the same lung or in both lungs. Thus, many of these patients would not have had resection if the usual criteria for operability had been used.

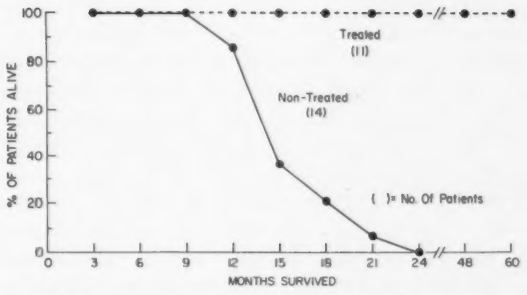

FIGURE 3.—Cumulative survival following onset of pulmonary metastasis in patients with TDT >40 days.

TABLE 2.—Summary of six treated patients with TDTs >40 days

Primary malignancy	Interval (mos) between primary tumor and metastases	Status of pulmonary metastases	Type of lung resection	Disease-free period (mos)	TDT (days)
Osteogenic sarcoma (right leg)	12	Solitary nodule, left; multiple nodules, right	Staged thoracotomies; multiple wedge resections, left and right	15	58,62
Osteogenic sarcoma (right leg)	7	Solitary nodule, right	Wedge resection, right	4	360
Liposarcoma (right leg)	1	Multiple nodules, right	Lobectomy, right	16	45
Synovial-cell sarcoma (right leg)	70	Solitary nodule, left; multiple nodules, right	Staged thoracotomies with lobectomy, left	62	78,80,92
Fibrosarcoma (right arm)	6	Multiple nodules, left and right	Bilateral thoracotomy; multiple wedge resections, left and right	9	45,46, 154,340
Fibrosarcoma	59	Solitary nodule, right; multiple nodules, left	Staged thoracotomies; wedge resection, right; lobectomy, left	35	54,72, 100

DISCUSSION

The incidence of pulmonary metastatic disease after apparent control of the primary malignancy varies from 10% to 30% (3,4). Over the past 20 years reports of operative treatment for metastatic cancer have become increasingly more frequent. The criteria for the selection of operative candidates have been discussed by several authors (5–10).

The tumor cell type and the origin of the primary malignancy have been suggested as critical factors (5,6). Some investigators feel that carcinomas have a better prognosis than sarcomas while others have the opposite opinion. However, long-term survivals have been reported with all types of lesions (7,8). Also, all sarcomas can not be grouped together as is often done since their behavior is unpredictable, and certain cell types such as fibrosarcomas and liposarcomas are known to have a better prognosis.

Other investigators have suggested that the interval between the primary and secondary operation is an important criterion for survival (9–12). However, Gliedman et al found no correlation between the length of this interval and the prognosis (5). Once again there are numerous instances where long-term survival occurs within a very short primary-secondary interval in nearly all reported series. In addition, a prolonged period of time between the primary operation and the appearance of pulmonary metastases does not guarantee long-term survival.

Multicentricity alone does not appear to correlate with prognosis. Although earlier reports stressed the poor prognosis associated with multiple nodules, Payne and associates (11) reported good results in selected patients with bilateral simultaneous pulmonary metastases. In a later series Thomford and associates (12) from the Mayo Clinic found that the 5-year survival rates for patients with solitary and multiple nodules were identical. Also, the presence of a solitary metastatic nodule does not rule out the presence of other foci too small to be clinically apparent. Other criteria for resection have included location of metastases, duration of symptoms, lymph node involvement, and extent of pulmonary resection. Recently, Feldman and Kyriakos (13) reported on a total of 40 patients with sarcoma metastatic to the lungs who were treated by pulmonary resection. Thirty-four of these patients were treated curatively, and six were treated palliatively. The cumulative 5-year survival rate in this series was 33.8%. Factors such as age, sex, and size of metastases (single or multiple) did not appear to influence survival.

As a result of these great variations among series, a more uniform and precise method is needed for comparison of tumor growth in each patient. The results of the studies by Collins et al (1,14) have led to the concept of the exponential growth of metastatic lesions. These observations have established that the majority of human metastatic tumors grow at a relatively constant rate or "doubling time." This concept has proven applicable not only for metastatic tumors, but also for various primary malignancies (15–17) and experimentally induced neoplasms (18).

Because of this pioneering study, Collins was able to show that a specific rate of growth was characteristic of an individual tumor—regardless of the pathologic diagnosis. He assigned a numerical value (in the form of "tumor doubling time") as a measure of growth in 24 patients with metastatic pulmonary disease based on serial radiographic measurements (1). Schwartz (19) confirmed this method in an additional eight patients with lung metastases, as well as proving its mathematical accuracy. Later, Collins extended this concept to a wide variety of lesions that produce pulmonary metastases and also showed that multiple pulmonary metastases will grow at similar fixed growth rates (14,20).

Our own results suggest that the tumor growth rate may vary for each patient depending upon the interrelationships between the host-immune defense mechanisms and the intrinsic growth potential of each tumor. We have found that the behavior of the metastatic lesions correlates well with the TDT calculated for each patient. Among three groups of patients, those with a longer doubling time had a longer interval of time between their primary tumor and metastases. There was little variation in the interval between the discovery of a metastatic focus and the appearance of multiple lesions. This would be expected because

all tumor foci had to occur before resection of the primary tumor, and since the metastatic tumor nodules grow at approximately the same rate, they should all become detectable on chest roentgenograms at approximately the same time. The survival rate correlated well with the TDT in the inoperable group. Those patients with a shorter TDT, and consequently a more rapid rate of tumor growth, have less chance for long-term survival. This was true although there was some variation in the interval between primary malignancy and metastases and in the number of metastatic foci.

In contrast to previous criteria that have been suggested as useful in evaluating operability of pulmonary metastatic disease, the TDT appears to enable one to select a group of patients for pulmonary resection who will have a more favorable prognosis. The particularly positive results obtained in those individuals with a TDT greater than 40 days occurred even though the majority had bilateral disease or multiple lesions in one lung (fig 4). Staged bilateral thoracotomy or even pneumonectomy did not appear to decrease the patients' chances for survival. Though previous criteria for resection were not followed, the greatly enhanced survival rate stresses that behavior of a metastatic lesion, operability, and chance of survival are more functions of the TDT than functions of other factors.

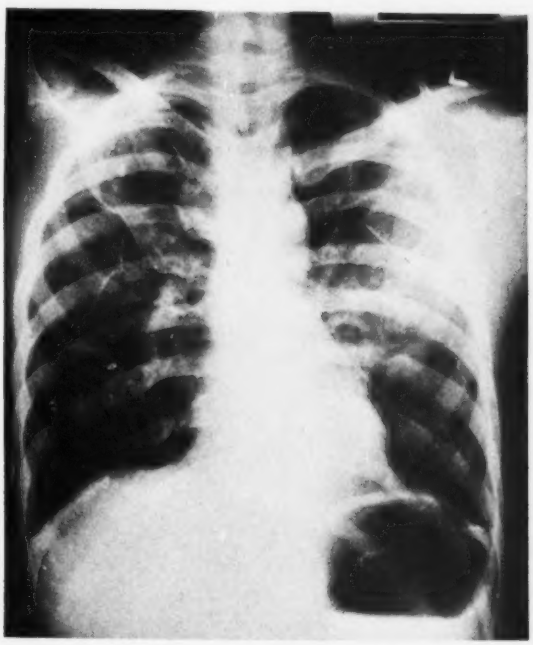

FIGURE 4.—Chest roentgenogram taken at 15 months after resection of 19 metastatic nodules in both lungs in an 18-year-old boy with fibrosarcoma from the left arm. Metallic structures seen in both lung fields are staples used at time of wedge resection.

1. Control of primary lesion
2. Metastasis limited to lungs
3. Resectable pulmonary mass
4. Suitable clinical and respiratory status
5. TDT > 40 days

FIGURE 5.—Indications for pulmonary resection of metastatic sarcoma.

Since surgical resection is the only hope for cure in pulmonary metastatic disease, a more aggressive approach should be undertaken in the treatment of patients with this disease. Our own indications for curative resection of pulmonary metastatic disease are listed in figure 5. These criteria include control of the primary lesion with the tumor confined to the lungs in a patient whose cardiorespiratory status is such that he can undergo thoracotomy. In addition, based on our present studies we would urge that all patients with metastatic pulmonary lesions that have a TDT greater than 40 days be considered for surgical resection even though lesions are multiple and bilateral.

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ANNOUNCEMENT

XIth International Cancer Congress

The Eleventh International Cancer Congress organized under the auspices of the UICC will be centered in Florence, Italy (October 20–26, 1974). Following the opening ceremony at the Palazzo Vecchio, the first 2 days will be devoted to special conferences organized mainly on a unidisciplinary basis, in Florence and in nearby towns (Montecatini, Lucca, Siena, Pisa, and Perugia). During the following days ordinary sessions of the Congress will be held in Florence itself. The programme comprises multidisciplinary symposia in the mornings and workshops or presentations of proffered papers in the afternoons. A memorial lecture will be delivered at the closing ceremony. Advanced courses in clinical oncology are scheduled to follow the Congress. The projection of scientific films and the display of scientific and commercial exhibits are planned. Forms for registration and hotel accommodations as well as information on travel, tours, and special events can be obtained by writing to:

General Secretariat
XI International Cancer Congress
Via G. Venezian, 1
20133 Milano, Italy

Dates

Opening ceremony: October 20 (Sunday afternoon)
Special sessions: October 21 and 22 (Monday and Tuesday)
Ordinary sessions: October 23–26 (Wednesday to Saturday)
Closing ceremony: October 26 (Saturday)
Advanced courses: October 28 and 29 (Monday and Tuesday)

CORRECTION

Due to a typesetting error, one line of the article "Murine Neuroblastoma: A Model of Human Disease" which appeared in the Nov/Dec issue (Vol. 57, No. 4) is incorrect. The first sentence of the last paragraph on page 411 should read as follows: "Since more than 50% of the patients with neuroblastoma have disseminated disease at the time of original diagnosis, chemotherapy provides the main hope for improvement in the prognosis of this disease."

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